

**AY-WB phytoplasma manipulations of host and  
non-host leafhopper interactions ©**

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Here is a male *Macrosteles quadrilineatus* waving his left foreleg, as he stridulates to other leafhoppers on an *Arabidopsis thaliana* leaf.

*M. quadrilineatus* show no reluctance to posing for a camera.

This photograph was taken by Andy Davis in the insectary at the John Innes Centre, Norwich, UK.

### **Abstract**

Phytoplasmas are plant pathogenic bacteria that are vectored by some Hemiptera. In plant hosts, phytoplasmas induce physiological changes and in both hosts modulate plant-insect interactions. Previously, interactions have been examined with both hosts infected with phytoplasmas. Thus, it is unclear which organism the effect stems from or how phytoplasmas facilitate changes. To investigate phytoplasma manipulations of insect-plant interactions, the model *Arabidopsis thaliana* was used together with the fully sequenced Aster Yellows phytoplasma strain Witches' Broom (AY-WB) and vector leafhopper *Macrostelus quadrilineatus*. I demonstrate possibility to differentiate effects of phytoplasma infection within plant and within insect hosts. To assess root cause of changes, AY-WB secreted effector proteins were examined, their roles within plants, and in manipulations of vector fecundity. One of the 56 secreted AY-WB proteins (SAPs) identified, SAP11, carries a nuclear localization signal and accumulates in plant cell nuclei (Bai *et al.* 2009). SAP11 is shown to reduce production of plant defense hormone jasmonic acid (Sugio *et al.* 2011). Stable expression of *SAP11* and 3 other SAPs in *Arabidopsis* increase fecundity of *M. quadrilineatus*.

In addition, phytoplasmas are known to affect non-host insect-plant interactions. Using the same approach, I demonstrate *D. maidis* survives and produces nymphs only on AY-WB-infected *Arabidopsis*. Furthermore, I show that whilst SAP11 has no effect on *D. maidis* survival, 3 other SAPs increase *D. maidis* survival and oviposition. These data suggest phytoplasmas utilize a suite of effector proteins to manipulate both host and non-host insect-plant interactions. Thus, AY-WB effector functions extend beyond direct interaction with plant hosts; they stimulate generation of insect vectors, and increase chance of uptake by novel insect hosts. This project highlights the value of using a model system in studying phytoplasma manipulation of their hosts and gives insight into development of evolutionary associations between phytoplasmas and vectors.

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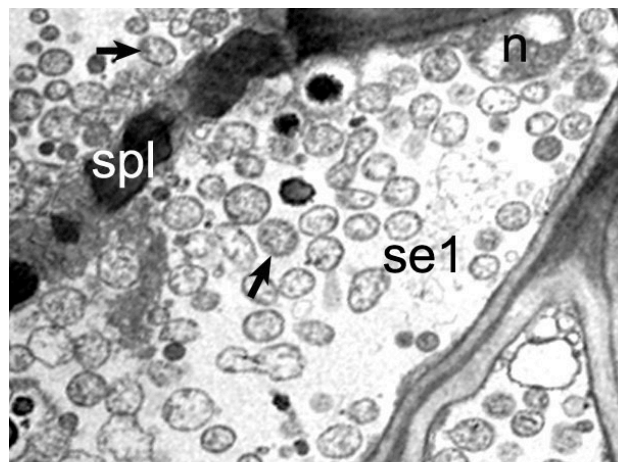
## **Introduction**

### *1.1 Phytoplasmas cause economic losses worldwide*

Phytoplasmas are pathogenic bacteria that cause damage to crop and ornamental plants worldwide. Despite the susceptibility of many hundreds of plant species around to infection, phytoplasma distinction from other plant diseases was made relatively recently (Doi *et al.* 1967). Phytoplasmas cause significant yield losses to a wide range of economically important crops, such as potatoes, maize and rice. Fruit such as pear, apple, plum, peach and vines, and large trees such as elms can also be infected. A graphic example is that of 'coconut lethal yellowing' where plantations in tropical regions can be reduced to a plantation of standing "telephone poles" by this phytoplasma infection (Fig. I.1.1.). Coconut lethal yellowing phytoplasma dramatically affects the livelihoods of coconut producers in the Caribbean, Mexico, Togo, Nigeria and Ghana in West Africa, East African countries, and also Asia and the far east (Mpunami *et al.* 1991). Symptoms of phytoplasma infection are yellowing of the plant tops, proliferation of young shoots (witches' broom), flowers becoming leafy (phyllody), greening of flower tissues (virescence) and bolting (elongation of stem and upper plant parts). Symptoms ultimately worsen over time until plant death. The latent period between inoculation and symptom development is dependent on the plant and phytoplasma species. Phytoplasmas are restricted to plant phloem (Figure. I.1.2.) and are particularly abundant in the plant's sink tissues.



**Figure. I.1.1. Coconut lethal yellowing phytoplasma.** Coconut lethal yellowing phytoplasma causes significant economic losses to coconut production in Caribbean Asia and African countries. Photo taken by Dr. Hubert de Franqueville, phytopathologist, CIRAD. [www.plantapalm.com/vpe/pestsndiseases/lyd\\_1a.JPG](http://www.plantapalm.com/vpe/pestsndiseases/lyd_1a.JPG) (sourced 20/3/08)



**Figure. I.1.2. Phytoplasmas are restricted to the sieve cells within the phloem tissue of infected plants.** Sieve pores allow phytoplasma movement between cells. Figure taken from Hogenhout *et al.* 2008, microscopy carried out by El D. Ammar at OARDC Ohio State University.

The extent of loss in crop yield due to phytoplasma infection is felt across the world. Many vineyards in Europe, South America, Australia and USA and Canada are reporting losses to phytoplasmas such as *flavescence dorée*, *boir noir*, *stolbur* and *grapevine yellows* (Beanland

*et al.* 2006; Sousa and Pereira. 2010; Zwolinska *et al.* 2010; Šeruga Musić *et al.* 2010; Gajardo *et al.* 2009; Padovan *et al.* 1996, Olivier *et al.* 2008). In China, Wheat blue dwarf phytoplasma has a devastating effect on wheat crops (Wu *et al.* 2010).

Symptoms of phytoplasma infection in many crops are similar to many virus infections, which has led to confusion in the past. For example grapevine yellows phytoplasmas (GYs) and grapevine leaf roll associated viruses (GLRaV) induce similar symptoms in infected vines, of downward rolled leaves, wrinkled dry berries and reduced sugar content (Martini *et al.* 1999; Boudon-Padieu 2003; Matus *et al.* 2008). Molecular distinction and diagnosis of phytoplasma over virus infection is made using polymerase chain reaction (PCR) amplification, of phytoplasma genes in infected plant tissue.

### *1.2 Classification of phytoplasmas is complex*

A Japanese research group first described Phytoplasmas as “mycoplasma like organisms” 40 years ago, after microscopic observations of infected plant tissue (Doi *et al.* 1967). These bacteria cannot be cultured in artificial medium outside their plant and insect hosts and were largely identified by the symptoms caused and crop plant host. This has limited the number of research groups investigating fundamental aspects of phytoplasma biology. Now diagnosis, identification and phylogenetic analyses are made with molecular tools. Whilst diagnosis of phytoplasma infection can be made using universal primers P1, P7 to amplify the 16S rDNA gene, specific phytoplasmas can be found by using known primers that amplify a specific region of the 16S rDNA gene (Zhang *et al.* 2004; Hogenhout *et al.* 2008). Specific phytoplasmas are also identified using sequencing and restriction fragment length polymorphism (RFLP) analyses of 16S (Lee *et al.* 1998; Fránová 2011), and the 23S rDNA, less conserved than the 16S, (Hoggetts *et al.* 2007; 2009). Furthermore, DNA barcoding of the 16S, and the elongation factor gene TU (*tuf*) of phytoplasmas is currently

being used to collate a database of sequences from which to identify phytoplasma strains (Contaldo *et al.* 2011).

Because phytoplasmas cannot be cultured, they are assigned *Candidatus* (*Ca.*) species names. A newly identified phytoplasma is a separate *Ca.* species when the 16S rDNA sequence has less than <97.5% sequence similarity to a previously named *Ca.* Phytoplasma species. However, in some cases phytoplasmas that have >97.5% similarity in the 16SrDNA sequence can still be assigned different *Ca.* species names, provided they have distinct plant host ranges and utilize distinct insect vectors (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group 2004; Bertaccini, 2007). Phytoplasmas are further distinguished and classified genetically by sequence differences in the spacer region between 16S and 23S, the 23S, *tuf*, *secA*, *secY* and ribosomal proteins (Lee *et al.* 1998; Seemüller; Contaldo *et al.* 2011). The use of different genes and methods provides various but similar phylogenetic analyses and classification of phytoplasma groups. To date, phytoplasmas have been separated into fifteen groups according to their 16S rDNA gene sequence. The Phylogenetic groups are outlined in table I.2.1 below (adapted from The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group. 2004).

General name	16S rDNA group	<i>Candidatus</i> ( <i>Ca.</i> ) Phytoplasma species	<i>Other described Ca. Phytoplasma species in each 16S rDNA group</i>
Aster yellows group	16SrI	<i>Ca.</i> Phytoplasma asteris	<i>Ca.</i> Phytoplasma japonicum AB010425 (Sawayanagi et al., 2007)
Peanut witches'-broom group	16SrII	<i>Ca.</i> Phytoplasma aurantifolia	
X-disease group	16SrIII	<i>Ca.</i> Phytoplasma pruni*	
Coconut lethal	16SrIV	" <i>Ca.</i> Phytoplasma	<i>Ca.</i> Phytoplasma

yellowing group		palmae"*	cocostanzaniae** X80117 <i>Ca. Phytoplasma</i> cocosnigeriae** Y13912 <i>Ca. Phytoplasma</i> castaneae AB054986 (Jung et al, 2002)
Elm yellows group	16SrV	" <i>Ca. Phytoplasma</i> ziziphi" (Jung et al, 2003)	" <i>Ca. Phytoplasma vitis</i> "* AF176319 " <i>Ca. Phytoplasma ulmi</i> " AF122910 (Lee et al, 2004)
Clover proliferation group	16SrVI	" <i>Ca. Phytoplasma</i> trifolii" (Hiruki and Wang 2004)	-
Ash yellows group	16SrVII	" <i>Ca. Phytoplasma</i> fraxini" (Griffiths et al, 1999)	-
Loofah witches'- broom group	16SrVIII	" <i>Ca. Phytoplasma</i> luffae"*	-
Pigeon pea witches'-broom group	16SrIX	" <i>Ca. Phytoplasma</i> phoenicium" (Verdin et al, 2003)	-
Apple proliferation group	16SrX	" <i>Ca. Phytoplasma</i> mali" (Seemüller and Schnieder 2004)	" <i>Ca. Phytoplasma pyri</i> " AJ542543, " <i>Ca.</i> <i>Phytoplasma</i> <i>prunorum</i> " AJ542544 (Seemüller and Schnieder 2004) " <i>Ca. Phytoplasma</i> <i>spartii</i> " X92869, " <i>Ca.</i> <i>Phytoplasma rhamni</i> " X76431, " <i>Ca.</i> <i>phytoplasma</i> <i>allocasuarinae</i> " AY135523 (Marccone et al 2004)
Rice yellow dwarf group	16SrXI	" <i>Ca. Phytoplasma</i> oryzae" (Jung et al, 2003)	-
Stolbur group	16SrXII	" <i>Ca. Phytoplasma</i> australiense" (Davis et al, 1997)	" <i>Ca. Phytoplasma</i> solani"* AF248959

Mexican periwinkle virescence group	16SrXIII	-	AF248960
BGWL group	16SrXIV	" <i>Ca. Phytoplasma cynodontis</i> " (Marccone et al, 2004)	-
" <i>Candidatus Phytoplasma brasiliense</i> " group	16SrXV	" <i>Ca. Phytoplasma brasiliense</i> " (Montano et al, 2001)	-

**Table I.2.1. Classification of phytoplasma phylogenetic groups.** As outlined by the IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group. 2004. This table has been simplified to highlight the main phytoplasma groups, their *Ca.* name and any additional *Ca.* phytoplasmas within groups.

\*Name proposed by the IRPCM Phytoplasma working team at the tenth International Congress of the International Organization of Mycoplasmology, Bordeaux 1994.

\*\*Name proposed by the IRPCM Phytoplasma working team at the fourteenth International Congress of the International Organization of Mycoplasmology, Vienna 2002.

- No *Ca. Phytoplasma* species described.

Even though no phytoplasmas have been cultured in cell free media as yet, four genomes have been fully sequenced and annotated. Comparisons of these and others that are near completion, have revealed similarities and differences in genomes, which indicate that phytoplasmas are evolving relatively rapidly (Hogenhout and Šeruga Musić 2010).

### *I.3 Metabolism of phytoplasmas*

Phytoplasmas are pleomorphic bacteria that infect and replicate in both plants and insect hosts. Phytoplasmas lack a cell wall and have one cell membrane. They are classed with other bacteria with similar morphological features into the Class *Mollicutes* (Razin *et al.* 1998).

Unlike other mollicutes, phytoplasmas cannot be cultured in artificial media because phytoplasmas gain much of their metabolic needs from their hosts (Gunderson *et al.* 1996; Bertaccini, Firrao *et al.* 2007). This is due to their limited metabolic capacities, as a result of extensive gene loss (Bai *et al.* 2006; Marcone *et al.* 1999; Oshima *et al.* 2004; Weisburg *et al.* 1989).

Spiroplasmas and mycoplasmas use glucose/fructose as a sugar source utilizing a phosphoenolpyruvate-dependant sugar phosphotransferase transport system (PTS). Achleplasmas and phytoplasmas have no known PTS system but use a maltose ABC transporter system, in which the maltose binding protein may also have an affinity for maltose, sucrose and trehalose (Bai *et al.* 2006; Razin, 2007). The latter is interesting as trehalose is the predominant sugar in insect haemolymph. All phytoplasmas identified so far are obligatorily transmitted by insects to plants. However, humans have manipulated transmission systems that allow transmission from plant to plant through grafting and through parasitic plants, such as dodder (Jarausch, *et al.* 1999). Among the other mollicutes, two *Spiroplasma* species, *S. citri* and *S. kunkelii* are insect-transmitted plant pathogens. *S. phoeniceum* is also a plant pathogen and thought to be insect-transmitted, but the insect vector for this spiroplasma has not yet been identified (Saillard *et al.* 1987; Hogenhout *et al.* 2008). The other ca. 30 described spiroplasma species are associated with insects and crustaceans, but not plants. Mycoplasmas are human and animal pathogens.

Mollicutes in general have limited metabolic capacities and have to uptake metabolites, including the majority of amino acids, from host cells. The phytoplasmas appear to possess fewer metabolic pathways compared to the spiroplasmas and mycoplasmas (fig. I.3.1) (Oshima *et al.* 2004). For example, phytoplasmas do not have any of the genes that encode the ATP synthase complex and lack more genes involved in DNA repair (Oshima *et al.* 2004 and Bai *et al.* 2006). So apparently, phytoplasmas will have to import ATP from host cells. This explains





glycerol-3-phosphate; DAG, diacylglycerol; AHHD, 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine; PRPP, 5-phosphoribosyl-1-pyrophosphate; *ugpABE*, ABC-type sugar transport system; *mgtA*, cation transport ATPase; *oppABCDF*, ABC-type dipeptide/oligopeptide transport system; *potABCD*, ABC-type spermidine/putrescine transport system; *cbiOQ*, ABC-type cobalt transport system; *mdlB*, ABC-type multidrug/protein/lipid transport system; *zntA*, cation transport ATPase; *phnL*, ABC-type uncharacterized transport system; PTS, phosphotransferase system.

Four phytoplasma strains have been sequenced to completion and all exhibit lack of ATP synthesis and are missing various elements of the glycolysis pathway, highlighted in orange above (Fig. I.3.1). However, there is variation between phytoplasma strains. For instance, *Candidatus* phytoplasma mali lacks the entire first section of the glycolytic pathway, whereas *Candidatus* phytoplasma australiense has 5 glycolytic genes and may have a functioning glycolysis pathway without the need for its lacking phosphotransferase system (PTS) (Tran-Nugyun *et al.* 2008; Kube *et al.* 2007; Seemüller and Schneider 2007).

#### *I.4 Phytoplasmas must replicate in insects and plants to disperse*

Phytoplasmas are able to invade and replicate in hosts belonging to two distinct kingdoms, plants (Plantae) and insects (Animalia). This live cycle that involves plants and insects is relatively unusual among prokaryotes, although there are more examples, such as *Spiroplasma citri* and *S. kunkelii*, and *Xylella fastidiosa* and *Ca. Liberibacter solanasearum*. Most of these are circulative bacteria within insect vectors and transmitted during salivation. Exceptions include *Xylella fastidiosa*, which is propagative in the insect foregut but non-circulative and relies on insect regurgitation for transmission to plants (Alves *et al.* 2008; Liefting *et al.* 2009; Breton *et al.* 2010; Hogenhout *et al.* 2008).

Any insect feeding on phloem of an infected plant can pick up phytoplasmas. The phytoplasmas are taken up along with the phloem nutrients and enter the insect oesophagus, anterior midgut and digestive tract. Some phytoplasmas may be broken down and passed on

for defecation, whereas other phytoplasmas are capable of attaching to and invading cells of the insect digestive tract. The latter phytoplasmas are thought to move in between the microvilli of gut epithelium cells and make their way through the cell into the space between the epithelial cell and the muscle cell wall. They may replicate at high numbers in gut epithelial cells and/or invade gut lining muscle cells where they can also replicate at high numbers (Fig. I.4.1). Phytoplasmas leave the cell through an exocytosis-like mechanism or the cells may lyse. Phytoplasmas may move through the basal lamina that aligns the epithelial cells at the haemolymph site, through mechanic disruption or by squeezing themselves through its 10nm pores (Christensen *et al.* 2004). It is striking that *Plasmodium* spp., which are protozoans (unicellular eukaryotes), also accumulate at high numbers at the haemolymph site of the mosquito gut before moving into the haemolymph (Billingsley 1998). It is possible that this space between the gut cells and the basal lamina provides a protected nutrient rich niche that is relatively free of the insect (innate) defense response components.

Once in the haemolymph, phytoplasmas can migrate to the salivary glands where they congregate in specific lobes (Lefol *et. al.* 1994., Nakashima and Hayashi. 1995., Ammar and Hogenhout. 2006). The capability of phytoplasmas to infect the insect salivary glands is important as these glands provide access to the plant phloem. Once the phytoplasmas pass through insect salivary gland cells and reach the saliva, they can be introduced into the plant phloem during insect salivation. In plants, the phytoplasma remains predominantly in the phloem tissue and moves from sieve cell to sieve cell by squeezing themselves through the pores of the sieve plates (Whitcomb and Tully. 1989). As phloem nutrients are generally directed towards the sink tissues of the plant (flowers, new leaf and stem growth and roots) this is where the phytoplasmas become concentrated. Note that symptom development in phytoplasma-infected plants is particularly prevalent in young plant tissues that are sink areas.



image adapted from file obtained from University of Wisconsin  
<http://wistep.wisc.edu/researchstudents.html> (sourced 10/10/2011).

### *1.5 Identifying phytoplasma vectors*

Only psyllids, leafhoppers and planthoppers have been found to vector phytoplasmas to plants. These are morphologically similar insects, belonging to two distinct suborders of the order Hemiptera; Auchenorrhyncha and Sternorrhyncha. [The Hemiptera were previously separated into two suborders, Homoptera and Heteroptera, however the suborder Homoptera was recently shown to be paraphyletic, thus, Hemiptera are now split into 4 suborders; Auchenorrhyncha, Sternorrhyncha, Heteroptera, and Coleorrhyncha (Von Dohlen and Moran 1995)]. Hemiptera are hemimetabolous insects, meaning they go through incomplete metamorphosis during their life cycle. These insects hatch from eggs as 1<sup>st</sup> larval instar and molt up to five times as they grow over a series of larval instars. Their morphology remains similar with merely the addition and growth of wing buds. Their last molt is into their adult form. There are about 100 known species of phytoplasma vectors out of 100,000 leafhopper species, 9000 planthoppers and 3850 psyllids described worldwide (Deitrich 2005; Wilson and Weintraub 2007; Wilson 2005; Bressan *et al.* 2009; Weintraub and Beanland 2006; Aléné *et al.* 2011)

Identification of a psyllid, leafhopper or planthopper that is a vector of phytoplasma is frequently complex, especially in woody crops that have dormant tissues and require a longer time for symptoms to develop. Indeed, at the time symptoms are appearing, the insects may have left. Further complication is that any insect herbivore feeding on phloem of infected plants will acquire the phytoplasmas but may not be able to transmit them. Thus, insects that are positive for phytoplasma presence in diagnostics assays are not necessarily vectors (Weintraub and Beanland, 2006). This complication requires several analyses, including transmission assays to confirm whether the infected insects can actually introduce phytoplasmas. In addition, microscopic localization of

phytoplasmas within insect organs can allude to vector capacity, phytoplasma must be in the salivary glands or saliva to be transmitted.

#### *1.6 Hemipterans can be very effective phytoplasma vectors - The role of saliva.*

Leafhoppers, planthoppers and psyllids have the potential to be efficient vectors of phytoplasmas for various reasons. Firstly, whilst their salivary properties are not fully known, it is understood that they use different types of saliva while feeding. Gelling saliva is secreted with initial stylet penetration and probing around plant cells, this hardens into a sheath to protect stylets (the sheath is left behind after the insect has finished feeding). This gel saliva sheath protects stylets from plant phenolic compounds that may accumulate due to cell damage. Proteins in the gel saliva sheath, such as catechol oxidase in aphids, are known to oxidize plant phenolic compounds (Hattori *et al.* 2005). Subsequently watery saliva is used whilst piercing the phloem sieve elements to feed on phloem (Ammar, 1986 and Moran, *et al.* 2002). It is thought that leafhoppers, planthoppers and psyllids have similar proteins in their watery saliva to those found in aphids, to enhance feeding or evade detection whilst discretely feeding on phloem in sieve tubes (Bos *et al.* 2011; Will *et al.* 2007). Indeed, salivary proteins of insects that feed on blood of vertebrate animals, such as mosquitoes and ticks, play an important role in suppressing vertebrate defense responses such as coagulation of blood (Caljon *et al.* 2006; Billingsley 1998). Some aphids are known to use calcium binding proteins in their watery saliva secretions that inhibit the plant occlusion response in sieve tubes, that block the flow of phloem between cells (Will *et al.* 2007). Will *et al.* showed that *Acyrtosiphon pisum* (Harris) green variant, and red variant, *Megoura viciae* (Buckton), *Aphis fabae* (Scopoli), *Myzus Persicae* (Sulzer), *Macrosiphom euphorbiae* (Thomas), *Rhopalosiphom padi* (Linnaeus), and *Schizaphis graminum* (Rondani) all stop feeding and

produce watery saliva containing calcium binding proteins to stem blockage of sieve tubes when occlusion was experimentally induced. Interestingly, the homology of salivary proteins between the 8 aphid species was less than 50% (Will *et al.* 2009). Furthermore, Mutti found that the salivary protein C002 was essential for *A. pisum* effective feeding. *A. pisum* aphids with knocked down production of C002, exhibited altered feeding behaviour and spent relatively less time feeding from phloem (Mutti *et al.* 2008). In addition, a homologue of C002 protein was found in *Myzus persicae*; MpC002. When MpC002 is knocked down in *M. persicae* using RNA interference, aphid offspring numbers were significantly reduced (Pitino *et al.* 2011).

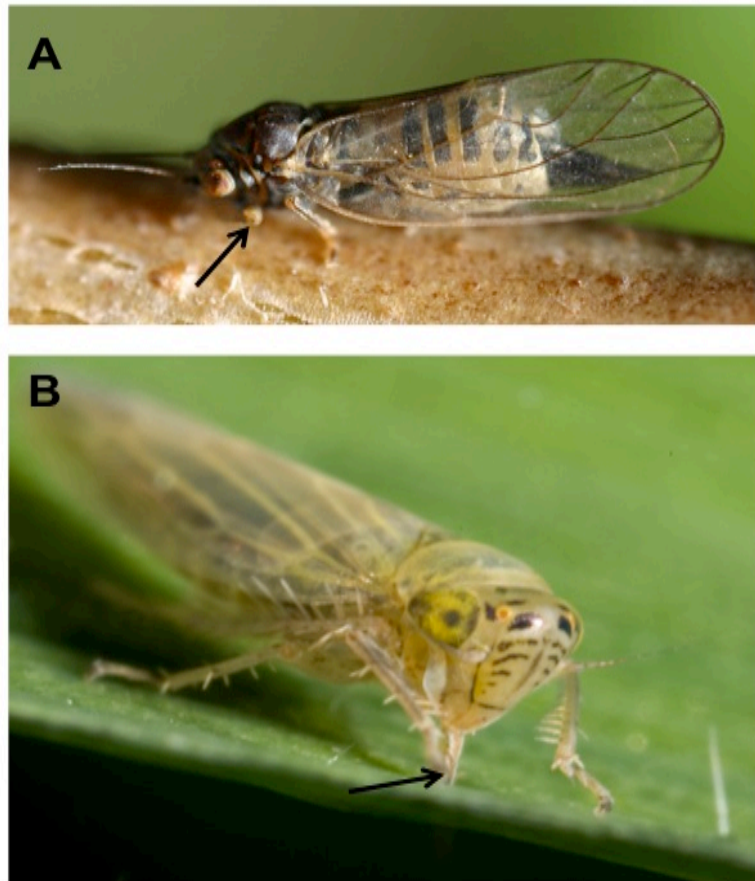
Some Hemiptera adopt relatively less discrete feeding behaviour and in many cases cause direct damage to crops independently of any pathogens they may transmit. The green rice plant hopper *Nephotettix cincticeps* is a more destructive feeder, puncturing epidermal and mesophyll cells on route to phloem. This planthopper produces laccase potentially to rapidly oxidize phenolic substances released from ruptured cell vacuoles, such as flavone, a carlinoside found in rice (Hattori *et al.* 2005). It is very likely that similar salivary proteins are found in all planthoppers, leafhoppers and psyllids to help them evade or down-regulate elicitation of a defense responses in their host plants (Ammar, 1986; Will *et al.* 2007). Thus the saliva proteins enhance the feeding of these insects, but also have the potential to aid effective transmission of phytoplasmas.

#### *1.7 Hemipterans can be very effective phytoplasma vectors - The role of stylets.*

The physiology of Hemiptera gives them the common name of the true bugs. These insects have sucking mouthparts with a long proboscis under the head and slender stylets protected by a labium sheath. Some examples of insect vectors of phytoplasma include; *Dalbulus maidis* is a leafhopper vector of Maize bushy stunt phytoplasma (MBSP) (Ammar and Hogenhout, 2006). *Cacopsylla melanoneura* is a psyllid vector of



*Candidatus* (Ca.) *Phytoplasma mali* (apple proliferation phytoplasma) (Tedeschi *et al.* 2006). The planthoppers *Hyalesthes obsoletus* and *Pentastiridius beieri* are vectors of many stolbur phytoplasmas (Gatineau *et al.* 2001).



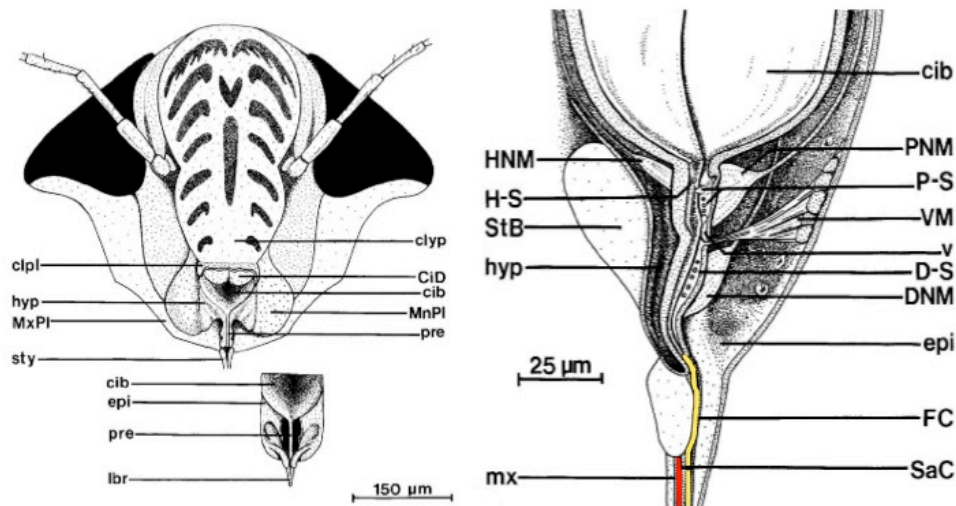
**Figure. I.7.1. Hemiptera use stylets within a proboscis to pierce and feed from plant vascular tissue.** The psyllid *Cacopsylla melanoneura* (A) and the leafhopper *Macrosteles quadrilineatus* (B) position their proboscis on the surface of epidermal cells and their stylets maneuver between mesophyll cells to the sieve elements, which they pierce to feed on phloem. Photographs highlight the proboscis position whilst feeding (arrow). Photo A adapted from The Natural History Museum Basel, <http://www.nmb.bs.ch/forschung/forschung-biowissenschaften/projekt-apfeltriebsucht.htm>. Photo B taken by Andy Davis, John Innes Centre, Norwich, UK.

The discrete feeding of these insects involves suction of plant liquid or blood depending on their host species. An example of an economically important blood feeding Hemipteran vector is *Triatoma infestans* the vector of *Trypanosoma cruzi*, chagas disease (Assumpção *et al.* 2008). The feeding apparatus of hemipterans is finely adapted to feed



efficiently on their appropriate host. On plants, Hemipterans position their probosci, and maneuver stylets between plant epidermal and mesophyll cells to reach phloem (or xylem) to feed. In minimizing damage to the cells, there is minimal arousal of host defenses. Stylet morphology, chemosensory capabilities and saliva properties contribute to the feeding behaviour and effective transmission of phytoplasmas.

Psyllids, leafhoppers and planthoppers have specialist feeding stylets in 2 canals that help them to assess the suitability of food and feed. A food canal is found to the left of the salivary duct within the maxillary stylet. The stylets act as straws, sucking up phloem or spitting saliva. A precibarium (a chamber of the foregut) is at the base of the stylets, this is lined with many chemosensilla. The chemosensilla are (chemical sensory hair-like structures linked to neurons) have paired dendrites that protrude into each of the two stylet canals. These dendrites sense the direction, force and pressure of the stylets, together with the chemical properties of the phloem. To do this effectively, the dendrites are in openings in the cuticle lining of the stylets, laid in the direction of fluid flow, which optimizes contact with the phloem passing through. Therefore, the chemosensilla and their paired dendrites provide the sensory system of the stylets. The chemosensilla are in two forms; a distal group (D-sensilla) and a proximal group (P-sensilla). The D-sensilla are on the epipharynx close to the divergence of the stylets and the P-sensilla are located on the epipharynx and also on the hypopharynx. The D-sensilla possibly act as first level discriminators of phloem compounds and the P-sensilla may allow further detailed sensing of compounds (Backus and McLean, 1982). The precibarial valve separates these two groups of sensilla (Fig. 1.6.2.). This valve is thought to be used when negative stimuli is felt by the chemosensilla. The valve can be used to expel phloem not to the leafhopper's taste as its stylets are withdrawn (Backus and McLean, 1982).

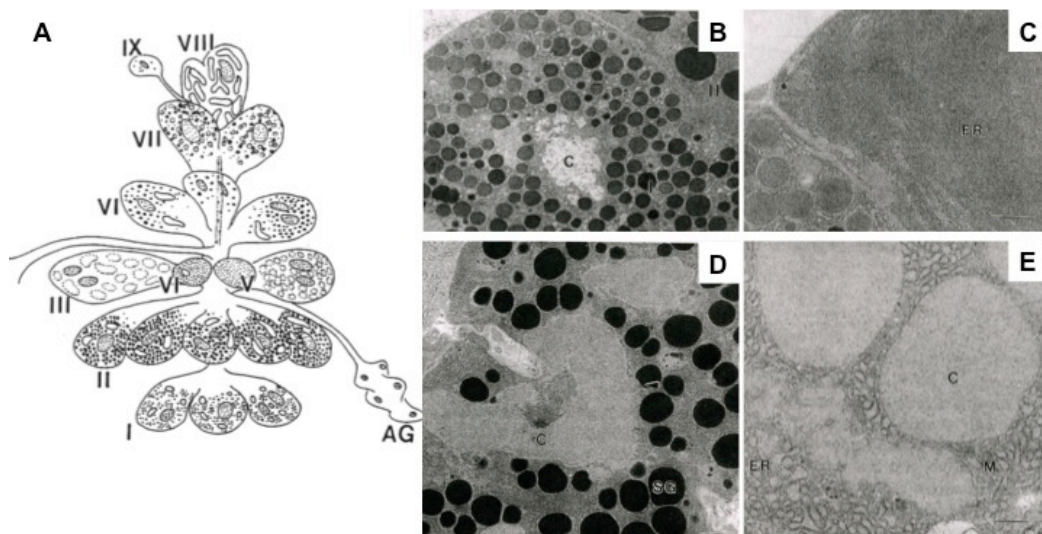


**Figure. I.7.2. The feeding apparatus of psyllids, leafhoppers and leafhoppers is well equipped to feed and efficiently transmit phytoplasmas.** The feeding apparatus of the Aster leafhopper *Macrostes fascifrons* (widely re-classified as *M. quadrilineatus*) is very sophisticated. Taken from Backus and McLean, 1982. Abbreviations: a, axon; cib, cibarium; CiD, cibarial diaphragm; clpl, clypellus; clyp, clypeus; cu, cuticle; d, dendrite; D1, D-sensillum No. 1; DNM, distal nerve cell mass; D-S, D-sensilla; epi, epipharynx; FC, food canal (shown in yellow); **GC**, glial cell; GNU, glial cell nucleus; HNM, hypopharyngeal nerve cell mass; H-S, H-sensilla; hyp, hypopharynx; Lap, lateral processes of cuticular sheath; lbr, labrum; LS, lymph sinus; m, mitochondrion; mn, mandibular stylet; MnP1, mandibular plate; MnR, mandibular stylet receptacle; mtu, microtubules; mu, muscle; mx, maxillary stylet; MxP1, maxillary plate; MxR, maxillary stylet receptacle; NeL, neural lamella; p, precibarial pit; P1, P-sensillum No. 1; PNM, proximal nerve cell mass; PNu, perikaryon nucleus; pre, precibarium; PrP, protoplasmic processes; P-S, P-sensilla; Sac, salivary canal (shown in red); SCM, sheath cell membrane; SL, sensillum liquor; SpD, septate desmosomes; StB, stylet base; sty, stylets; to, tonofibrillae; **v**, precibarial valve; VM, precibarial valve muscle.

The sensory system within stylets and the proboscis, together with muscles and valves that enable the manipulation of the stylets, provide hemiptera such as leafhoppers, planthoppers and psyllids precision equipment for detecting and feeding on their chosen hosts.

*I.8 Hemipterans can be very effective phytoplasma vectors - The role of salivary glands.*

Linked the stylets via the salivary duct are the salivary glands, consisting of principle and accessory glands. The principle gland is made up of 8 follicles each with its own ductile. These ductiles, and that of the duct of the accessory gland come together into one principle duct located in the maxillary stylet (Wayadande *et al.* 1997). The maxillary stylet is consequently used in salivation of saliva during feeding. During feeding these insects salivate almost constantly, thus, phytoplasmas are efficiently transmitted from infected follicles via the salivary duct network. Each of the salivary gland follicles is distinct in morphology to varying degrees (Fig. I.6.3). Variation in physiology leads to the hypothesis that each salivary follicle performs a different function in the manufacture of saliva. Whether they each produce a different type of saliva or contribute different compounds to make up saliva is not known (Ammar, 1986). Thus far only two distinct types of saliva have been identified, as described above. The gel sheath saliva and the water saliva produced in the follicles could account for some morphology differences in follicles. It is likely that on dissection and study of specific follicles, specificity in protein production will be discovered (Funk 2001; Hattori *et al.* 2005; Cherqui and Tjallingii, 2000).



**Figure. I.8.1. Salivary gland follicles of Hemiptera exhibited varied morphology.**

This variance in morphology suggests different follicles contribute different ingredients to insect saliva. The diagram of one side of paired salivary glands of *Dalbulus maidis* (A) highlights the variance in granular density of the individual lobes. Photos of the ultrastructure of *D. maidis* salivary gland cell types demonstrate the different cell structures in individual follicles (B-E). B is follicle I and II; C, higher magnification of type I highlighting large area of endoplasmic reticulum; D, type II with large secretory granules and large canaliculi; E, type III with no secretory granules and large canaliculi. Taken from Wayadande *et al.* 1997. Abbreviations; I-X, follicles of type I-IX; AG, accessory gland; C, canaliculus; ER, endoplasmic reticulum; M, mitochondrion; SG, secretory granule.

The complexity of leafhopper, psyllid and planthopper salivary glands facilitates the variety of saliva production. This, alongside specialist morphology of the mouthparts, and discrete feeding behaviour make psyllids, leafhoppers and planthoppers potentially very effective vectors of plant viruses and phytoplasmas.

*1.9 Plants defend themselves against insect herbivores with secondary metabolites*

In plants, the jasmonic acid (JA) pathway is often activated when insects feed on plant tissues (Abe *et al.* 2008; Devoto *et al.* 2008; Diezel *et al.* 2011). When phloem feeders such as leafhoppers and aphids feed on plant phloem, the JA pathway is only minimally induced initially (Maffei *et al.* 2007; Kessler *et al.* 2004). This is explained by the different feeding mechanisms adopted by different insects, for example caterpillars induce a lot of JA compared with aphids. Caterpillar chewing of leaf tissue is far more destructive than leafhoppers and aphids careful maneuvering of stylet between cells of plants. Leafhopper feeding is more discrete and comparable to, for instance, the penetration of biotrophic fungal hyphi and bacterial type three secretion pili. Fungal and bacterial biotrophs may induce genes involved in production of hormones including salicylic acid (SA), JA, ethylene or phytoalexins during the defense response of infected plants

(Hogenhout *et al.* 2008; Ferrari *et al.* 2007). Accumulation of these hormones may trigger the production of secondary metabolites in defence against herbivory. Interestingly aphids have not been shown to induce high levels of hormones including SA and JA (Maffei *et al.* 2007; Devoto *et al.* 2008). Moreover, whilst the initial plant response to aphids is small, some plants, in time induce the JA cascade to induce plant secondary metabolites or volatile production. Volatile production may attract predators of the discrete feeders (Girling *et al.* 2008). Specialist discrete feeders may have adapted to overcome their host plant defences whereas generalists may elicit a variety of plant defence responses from their various hosts.

#### *1.7 Phytoplasmas have mixed effects on their insect vectors*

Phytoplasmas infection has two potential impacts on psyllid, leafhopper and planthopper vectors. Infection may affect the internal biology, and/or the behaviour of the insect.

##### *Phytoplasmas effect on internal biology of insects*

As phytoplasmas enter the anterior midgut with a phloem meal, they must survive the pH, digestive enzymes, ionic strength, redox potential and peristalsis of the gut. In addition, they must invade gut epithelial cells either intracellularly or intercellularly by entocytosis or diacytosis. Many bacteria enter the gut and prompt an immune response, for example, gram-negative bacteria trigger an innate immune defence (Imd) response, and gram-positive bacteria trigger a Toll response. Both responses are initiated by recognition of bacterial peptidoglycans and cause the release of antimicrobial peptides (AMPs) such as cecropin or diptericin. Many pathogenic bacteria secrete virulence proteins (effectors) to breakdown or evade these antimicrobial peptides (Vallet-Gely *et al.* 2008). Phytoplasmas, with no cell wall, don't produce peptidoglycans (Sugio *et al.* 2011), furthermore, not all epidermal cells along the insect midgut initiate an immune response (Vallet-Gely *et al.* 2008). Phytoplasmas do produce cold shock protein

(CSP) and elongation factor Tu (EF-Tu), which trigger immune response in some plant hosts such as the *Brassicaceae* and *Solanaceae* (Sugio *et al.* 2011). The genes for CSP (CSP15) and EF-Tu (*tuf*) are highly conserved in bacteria so it is plausible that immune defences of some insects may be triggered by either of these. An immune response to EF-Tu is only so far recorded in *Drosophila* (Hofweber *et al.* 2005; Landini *et al.* 1992).

Thus, it is unknown whether phytoplasmas go undetected in vector insects by either; not eliciting an immune response, or by accumulating near to and invading epidermal cells that are immunologically deficient. If phytoplasmas do trigger an immune response via recognition of conserved proteins, they may secrete effector proteins to block or destroy insect anti microbial peptides. The phytoplasmas must then adhere to anterior midgut epithelial cells and invade them by entocytosis or diacytosis and pass through these cells to the area between the basal lamina (basement membrane) and plasmalemma (membrane above muscle cells) (Hogenhout *et al.* 2008; Bosco and D'Amelio 2010). Here, phytoplasmas replicate before passing through muscle cells to the haemolymph. It is unknown what immune response, if any, are triggered by these steps. *Spiroplasma citri* surface proteins facilitate adherence to the epithelial cells and receptor-mediated endocytosis (Kwon *et al.* 1998; Bosco and D'Amelio 2010). Phytoplasmas may use a similar strategy, flavesence dorée adheres to Nitro-celulose extracts of many insect cells, and onion yellows phytoplasmas antigenic membrane protein, Amp has been shown to associate with insect microfilament complex (Lefol *et al.* 1993; Suzuki *et al.* 2006).

Phytoplasmas may induce (and overcome) immune response at each step, trigger haemocytes and plasmatocytes (defence cells that perform phagocytosis) in fat-bodies of haemolymph, and repeat immune response induction on entering other organs such as salivary glands (Vallet-Gely *et al.* 2008). Phytoplasmas in great numbers may come at a defence cost to host insects, or indeed they may go largely unnoticed. So far the immune response of insects and their vector capacity has not

been fully studied. However, Bosco *et al.* showed that higher Chrysanthemum yellows phytoplasma multiplication in the insect correlates with higher vector efficiency in *Macrostelus quadripunctatus* compared with *Euscelis variegatus* and *E. incisus* (Bosco *et al.* 2007). Spiroplasma citri infection has a negative impact on the survival and fecundity of *Circulifer tenellus*. On examination of infected insect midgut lining muscle cells, Kwon *et al.*, found that heavily infected cells had disorganized endoplasmic reticulum and compromised muscle fibres (Kwon *et al.* 1998). The immune response of phytoplasma infection could be indicative of the vector capacity of the insect, and the evolutionary duration of the association.

Symbiotic bacteria of hemipterans may further complicate phytoplasma interactions with host insects. Symbionts in many cases provide protection from pathogenic invading bacteria, or transfer nutrients that the insects are unable to sequester themselves (Moran 2006; Ziente *et al.* 2001). Many psyllids and leafhoppers have long associations with symbionts such as *Sulcia muelleri* and *Hamiltonella defensa* in bacteriocytes in the midgut and fat bodies of haemolymph that defend against invading pathogens using toxins (Takiya *et al.* 2006; Moran *et al.* 2008). It is unknown whether phytoplasmas compete with or are controlled by primary (obligate on both parties, domesticated) bacterial symbionts such as *S. muelleri* or *H. defensa*, but competition does occur between secondary symbionts (facultative, bacteria must reside in insect for part of its life cycle). For example, corn stunt spiroplasma (CSS) competes with maize bushy stunt phytoplasma (MBSP) in the corn leafhopper *Dalbulus maidis*. Cross-protection occurs, if *D. maidis* acquires CSS and MBSP in either order, the insect will vector both to corn initially but MBSP efficacy will reduce over time, whilst efficacy of CSS transmission remain high. Thus, CSS may be out-competing with MBSP in invading host cells and replicating at a higher rate within the insect (Maramorosch, 1958 in Bosco and D'Amelio 2010). Phytoplasmas may compete with other phytoplasma strains for

invasion of host cells and replication, or the insect immune response may be permissive of invasion by one phytoplasma strain over another in multiple phytoplasma infection. Thus natural selection may occur in the insect dictating phytoplasma acquisition and transmission (Bosco and D'Amelio). Phytoplasma membrane interactions with host insect cells needs to be further explored to gain greater understanding of the evolution of phytoplasma associations with insect vectors.

#### *Phytoplasmas effect on behaviour of insects*

Some insect vectors phytoplasmas have exhibited higher survival rates or produce more offspring on their host plants such as the aster leafhopper, *Macrostelus quadrilineatus* (Forbes) when infected with aster yellows phytoplasma (AYPs) (Beanland *et al.* 2000; Nault and Ammar 1989; Murrall *et al.* 1996; Severin 1946). Interestingly, *M. quadrilineatus* show increased fecundity on plants infected with one isolate of AYP, but not with another AYP isolate (Peterson 1973; Beanland *et al.* 2000). Some insects have reduced survival when infected such as *Scaphoideus titanus* when infected with flavescence dorée, a 16SrV phytoplasma belonging to the elm yellows group, that causes a grapevine yellows (Bertin *et al.* 2007). These differences in effect on survival and fecundity could be indicative of the evolutionary duration of the vector association with the phytoplasma. Longer associations may have evolved to be beneficial to the vectors (Nault 1989; Purcell 1971). For example, interactions may come at little or no immune response cost to the insect, and the insect cells may have a permissive association with the phytoplasma strain that as such renders the phytoplasma as a facultative symbiont. This makes the assumption that the effect originates from infection within the insect, which may not be the case. Most studies into vector survival and fecundity examine infected vectors on plants that also become infected. This blurs any distinct effects that may originate in the insect, originate in the plant, or indeed have an additive effect.



In examining non-host insect-phytoplasma interactions, effects distinctly originating in plants have been observed. Phytoplasma plant host ranges are limited by insect vector plant host range (Hogenhout *et al.* 2008). Phytoplasmas may have adopted an evolutionary strategy in attracting novel insects as vectors. Some phytoplasmas have been reported to alter plant susceptibility to novel insect herbivores. For example, the maize specialist feeder, *Dalbulus maidis*, can survive on AYP-infected lettuce (*Lactuca sativa* L.) and China aster (*Callistephus chinensis* Nees), but not on healthy lettuce and China aster plants (Beanland *et al.*, 2002, Purcell 1973). In another case, apple proliferation phytoplasma (*Candidatus* Phytoplasma mali) infected apple trees give off modified volatiles, which attract vector psyllid, *Cacopsylla piri* (Mayer *et al.* 2007). Phytoplasma infection within the insect may have a direct effect inside the insect resulting in an altered behaviour. Alternatively, phytoplasma infection within the host plant may have an original effect on the plant, which in turn has a secondary effect on the behaviour of the insect. Further study of how phytoplasma impacts on insect-plant interactions will enhance our understanding of phytoplasma epidemiology.

#### *1.11 Understanding phytoplasma dispersal in crops requires knowledge of vector ecology and taxonomy*

Aster Yellows phytoplasmas (AYPs) collectively have a wide plant host range affecting more than 80 dicot and monocot plant species worldwide (Hogenhout *et al.* 2008). In the USA and Canada, AYPs most frequently reported vector is *Macrostelus quadrilineatus*, which has a wide plant host range (Wilson and Turner, 2010). *M. quadrilineatus* is a migratory leafhopper and its geographic range contributes to the dispersal of AYPs to crops. It is found in spring in the southern states of USA and migrates, with the aid of storm fronts, north as far as Southern Canada. However, *M. quadrilineatus* is one species of a complex of species first alluded to by Beirne 1952, who described three complexes

within the *Macrosteles* genus; variatus-, fascifrons-, and cristatus-complexes (Beirne 1952). The fascifrons-complex was further described by Vilbaste in 1980 and later by Hamilton in 1983, and again 1985 and 1988 as the quadrilineatus-complex (Tab. I.11.1). Complexes are a group of species within a genus, previously described as one species, later recognised as distinct but significantly similar species. The *Macrosteles* genus has four species complexes, of which the *quadrilineatus* complex is the largest. The *quadrilineatus*-complex encompasses 10 *Macrosteles* species in N. America and 1 in Europe, (Hamilton 1985), but possibly contains a further 18 species in N. America and 11 in S. and C. America (Kwon 1988).

Location	Species	Author	Date
<b>Trans boreal</b>	alpinus	Zetterstedt	1828
	bifurcatus	Beirne	1952
	borealis	Dorst	1931
	canadensis	Kwon	2010
	curvatus	Beirne	1952
	divisus	Uhler	1877
	elongatus	Beirne	1952
	fascifrons	Stål	1858
<b>Trans boreal</b>	fieberi	Edwards	1889
<b>Trans boreal</b>	frontalis	Scott	1875
	galeae	Hamilton	1987
	guttatus	Matsumura	1915
	inundatus	Hamilton	1987
	jussiaeae	Moore & Ross	1957
<b>Trans boreal</b>	laevis	Ribaut	1927
	lagus	Hamilton	1983
	lepidus	Van Duzee	1894
	lineatifrons	Stål	1858
	obsoletus	Dorst	1931
	pallidus	Osborn	1915
	potorius	Ball	1900
	*quadrilineatus	Forbes	1885

	scriptus	DeLong	1924
	*severini	Hamilton	1983
	skalkahiensis	Beirne	1952
	slossonae	Van Duzee	1893
<b>Trans boreal</b>	sordidipennis	Stal	1858
	tesselatus	Hamilton	1983
	urticae	Moore & Ross	1957
	vilbastei	Hamilton	1983
	vulgaris	Kwon	2010
	wilburi	Dorst	1937
<b>N. America</b>	30 new sp.	Kwon	1988
	18 of these in quadrilineatus-complex		
<b>C. &amp; S. America</b>	11 additional new sp. in complex found in		
<b>Europe</b>	lindbergi	Dlabola	1963

**Table. I.11.1. Thirty species of the *Macrosteles* genus are found in North America.** All species highlighted in yellow are members of the “quadrilineatus complex” described by Hamilton 1985 (green text) and Kwon 1988 and 2010 (black text). \*First four species found in Canada and described by Hamilton 1983 as the fascifrons-complex (later described as quadrilineatus-complex). \*\*\*New species described by Kwon 1988 in his PhD thesis, unpublished, 30 new species found in N. America, of which 18 he described as belonging to the quadrilineatus-complex. A further 11 species were also described by Kwon from S. & C. America as belonging to the same quadrilineatus-complex.

Dissection and examination of the abdominal internal apodemes (associated with sound producing) and genitalia of male leafhoppers distinguish species within the quadrilineatus-complex. Colouration, body size and wing morphology can all vary between populations and seasons of the same species (Kwon 1988; Hamilton and Kwon 2010a, 2010b; Claridge 1985). Distinction can also be made using analysis of leafhopper calls (Claridge 1985; Purcell and Loher 1976). In addition body size and wing morphology can be affected by my external factors, such as fitness and environmental conditions (Beanland *et al.* 2004; Larson *et al.* 1990; Purcell and Loher 1976; Kwon 1988). In light of the similarity of leafhoppers in this complex, there is the potential risk that

some confusion has occurred in identification of some vectors of AYPs, exacerbated by the lack of recent taxonomic publications regarding the quadrilineatus-complex. For example in Hawaii an outbreak of AYP in watercress was attributed to the accidental introduction of the vector *M. quadrilineatus* Forbes or *M. fascifrons* Stål in 2000. The leafhopper has since been identified as *M. severini* Hamilton, which was first described as a vector of AYP in 1987 (Oldfield 1987; Smith *et al.* 2002; Borth *et al.* 2006; Le Roux and Rubinoff 2008). The quadrilineatus-complex includes the species *M. quadrilineatus* and *M. fascifrons*, which in many texts are referred to as '*M. quadrilineatus* Forbes (formally *M. fascifrons* Stål)' which leads to ambiguity over which species is being discussed (Hamilton 1985; Kwon 1988; Weintraub and Beanland 2006; Goodwin *et al.* 1998; Lee *et al.* 2004). An extension of the confusion is that *M. fascifrons* may actually be *M. quadrilineatus* and *M. severini*, but with the inevitable loss of some old specimens, some taxonomists are currently looking for more examples of male *M. fascifrons* to compare with *M. quadrilineatus* and *M. severini* and others in the complex. To date, the theory stands that *M. fascifrons* populations may be distinct from other species within the complex, but that *M. quadrilineatus* and *M. severini* populations may have wrongly been identified as *M. fascifrons* in the past. The quadrilineatus-complex demands many more questions of the ecology of these insects and their vector capacity. For instance, it is likely that these insects diverged from one species through speciation as a result of population migration, host plant choice, and isolation. Further study of the geographical migration routes and host ranges of these insects will yield clues as to how this speciation occurred. It is also feasible the speciation of these insects has resulted in differences in permissiveness of phytoplasma infection. Many leafhoppers of the *Macrostelini* tribe have evolved to become virus and pathogen vectors (Sorensen and Sawyer 1989; Kwon 1988). Oldfield reports that *M. severini* is an efficient vector of AYPs but not spiroplasmas (Oldfield 1987). In total, 8 *Macrosteles* leafhoppers are recorded phytoplasma vectors (Tab. I.11.2). Only 3 of these species are

within the quadrilineatus-complex; *M. quadrilineatus*, *M. severini*, and *M. fascifrons*. Ecological study of the Macrosteles quadrilineatus-complex, their habitat and plant host ranges together with study of interactions with encountered phytoplasmas and (if any) and vector capacity, will yield clues into what renders *M. quadrilineatus*, *M. severini*, and *M. fascifrons* such efficient phytoplasma vectors.

Vector species	Phytoplasma	Plant host	Location	Author
<b><i>Macrosteles cristata</i> (Ribaut 1927)</b>	Clover phyllody/16SrI-C	Various families	Europe	Brcak 1979
	Clover dwarf/16SrIII-B	Vegetables	Europe	Brcak 1979
<b><i>M. laevis</i> (Ribaut 1927)</b>	European Aster yellows/16SrI-B	Various families	Europe	Brcak 1979
	Clover phyllody/16SrI-C	Various families	Europe	Valenta 1960
	Clover dwarf/16SrIII-B	Various families	Europe	Brcak 1979
	Stolbur/16SrXII-A	Tomato/Potato	Turkey	Guclu & Ozbek 1991
<b><i>M. quadrilineatus</i> (Forbes 1885) (***=<i>fascifrons</i> Stal 1855)</b>	American Aster yellows/16SrI-A	Vegetables	North America	Gilmer 1954
	European Aster yellows/16SrI-B	Vegetables	Europe	Esau 1976
<b><i>M. quadripunctulatus</i> (Kirschbaum 1968)</b>	Kok-saghyz yellows	Chrysanthemum	Europe	Brcak 1979
	Chrysanthemum yellows/16SrI-B	Chrysanthemum	Russia, Europe	Palermo <i>et al.</i> 2001
<b><i>M. severini</i> (Hamilton 1983)</b>	Aster yellows	Various families	North America	Hamilton 1983
	Aster yellows	Watercress	Hawaii	Heu <i>et al.</i> 2003
<b><i>M. sexnotatus</i> (Fallen 1806) (=<i>devastans</i> [Guerin-Meneville 1852])</b>	Lissers	Hyacinths, gladiolus	Europe	van Slogteren & Muler 1972
	Aster yellows	Flowers	Europe	Savio & Conti 1983
<b><i>M. striifrons</i> (Anufriev 1968) (=<i>orientalis</i> [Vilbaste 1968]) (=<i>plicativus</i> [Dubovski 1970])</b>	Anemone witches' broom	Various families	Japan	Kato <i>et al.</i> 1989
	Chrysanthemum yellows/16Sr-IB	Chrysanthemum	Europe	Palermo <i>et al.</i> 2001
	Eggplant dwarf	Solanaceae	Japan	Okuda <i>et al.</i> 1997
	Garland Chrysanthemum	Chrysanthemum	Japan	Shiomi & Sugiura 1984.
	witches'-broom/16SrI			
	Marguerite yellows	Chrysanthemum	Japan	Shiomi & Sugiura 1984.
	Mitsuba witches' broom/16SrI	Japanese honewort	Japan	Shiomi & Sugiura 1984.
	Onion yellows	Chrysanthemum	Japan	Shiomi <i>et al.</i> 2001
<b><i>M. viridigriseus</i> (Edwards 1924)</b>	Tomato yellows	Solanaceae	Japan	Kato <i>et al.</i> 1988
	Clover Phyllody/16SrI-C	Clover, plantain	England	Frazier & Posnette 1956
	Clover witches' broom	Clover, plantain	England	Frazier & Posnette 1956

**Table. I.11.2. Eight Macrosteles leafhoppers are known phytoplasma vectors.**

Adapted from Weintraub and Beanland 2006. \*\*\**M. fascifrons* may be a distinct vector species in addition to *M. quadrilineatus* or the vector may have been initially identified as *M. fascifrons* and subsequently identified as *M. quadrilineatus* (Kwon 1988; Hamilton and Kwon 2010).

*I.12 Model systems are invaluable in understanding phytoplasma dispersal by their vectors*

Using a model host plant, a known insect vector, and a fully sequenced phytoplasma to examine changes in insect-plant interactions, is essential to unravelling the complexities involved in phytoplasma dispersal. Known phytoplasmas and vectors are monitored around the world to warn farmers and improve control. Both migratory and sedentary vector species exhibit fluctuations in population numbers and infective individuals geographically, annually and through the season. Geographic dispersal of vectors can be weather dependent as many populations travel on storm fronts (Hoy *et al.* 1992). The fitness of the insect also affects the transmission of phytoplasmas. The age of *M. quadrilineatus* affects its transmission efficacy of AYP ‘Bolt’ and AYP ‘Severe’ (Murrall *et al.* 1996). Palermo *et al.* found that *M. quadripunctulatus* and *Euscelidus variegatus* lost infectivity for periods, and that AYP acquisition efficiency decreased with age (Palermo *et al.* 2001). The behaviour of the insect populations also affects the dispersal and likelihood of acquisition. Migrant insects movement between crops is dependent on plant species, timing of plant senescence, insect sex, and time of day (Maixner 2010; Todd *et al.* 1991; Beanland *et al.* 1999; 2005).

AYPs also affect population numbers and indirectly dispersal and proportion of infective individuals. *D. maidis* females infected with MBSP oviposit more and earlier than healthy females (Madden *et al.* 1984). Study of phytoplasma interactions within a model system enables clarification into how phytoplasma manipulations of plant hosts and insect vectors affect epidemiology of phytoplasmas and their vectors. Using a fully sequenced phytoplasma with a model plant and a known insect vector, allows examination of plant defences and interactions with phytoplasma secreted proteins. Phytoplasmas are known to have secreted proteins that probably

modulate their host environments and enable successful replication (Bai *et al.* 2006; 2009; Hogenhout *et al.* 2008; Hoshi *et al.* 2009). Four phytoplasmas have been fully sequenced to date; Onion yellows phytoplasma (OY-M *Candidatus* Phytoplasma asteris) (Oshima *et al.* 2004), Australian grapevine yellows (AUSGY; *Ca. P. australiense*) (Tran-Nguyen *et al.* 2008), Aster Yellows phytoplasma strain Witches' Broom (AY-WB; *Ca. P. asteris*) (Bai *et al.* 2006), and Apple Proliferation phytoplasma (AP; *Ca. P. mali*) (Kube *et al.* 2008). Secreted proteins described as candidate effector proteins were found encoded on all sequenced phytoplasma chromosomes. In OY-M there are 45 candidate effectors on chromosomes, in AUSGY, 41, in AP, 13, and in AY-WB, 49 (Bai *et al.* 2006; 2009; Kube *et al.* 2008; Hoshi *et al.* 2008; Oshima *et al.* 2004; Tran-Nguyen *et al.* 2008). These candidate effectors are hypothesized to modulate insect and plant host environments to enable survival, replication and dispersal. It is possible to examine phytoplasma candidate effectors impact on plant-insect interactions, using a model plant such as *Nicotiana benthamiana* or *Arabidopsis thaliana*.

### *1.12 Scope of thesis*

This project used a model system to further understand changes made by phytoplasmas to their hosts that in turn affect insect-plant interactions and phytoplasma epidemiology.

In preliminary studies, I found that *Arabidopsis* is susceptible host plant to both AY-WB phytoplasma and its vector *M. quadrilineatus*. AY-WB was isolated in Ohio (Zhang *et al.* 2004) *M. quadrilineatus* was identified as the vector in Ohio and colonies were established from leafhoppers in this area. *Arabidopsis* is also found growing in Ohio, but is not reported as a natural host for AY-WB or *M. quadrilineatus* (Clarke 1993). The *Arabidopsis* model plant has known genes for defence and plant development that can be further studied for their responses to phytoplasma infection. In addition, transgenic *Arabidopsis* can be generated to express genes encoding phytoplasma effector proteins, thus, eluding to possible function within the plant. *M. quadrilineatus* is

the widely known vector of AYP including AY-WB in North America. Furthermore, it may serve as a 'bench mark' insect for species within the quadrilineatus-complex and other *Macrosteles* species.

*M. quadrilineatus* is more successful on AYP infected aster and lettuce than healthy plants (Beanland et al. 2000; Ebbert and Nault 1998). The AYPs may either increase oviposition rate of insect directly or alter plant hosts in such a way that they are more suitable hosts. All studies to date have used AYP infected insects on plants that also become infected, thus blurring the origin of the effect.

In chapters one and two, I aim to highlight that it is possible to distinguish phytoplasma effects stemming from the insect and those stemming from the plant in insect-plant interactions. Furthermore, I will show that both effects have an impact in insect-plant interactions. AYPs have also been shown to alter non-host plant-insect interactions. For example, the maize specialist leafhopper *Dalbulus maidis* feeds and survives on AYP infected lettuce and aster but not on healthy plants (*D. maidis*).

In chapter three I show that AY-WB can alter the susceptibility of *Arabidopsis* to *D. maidis*, leading to increased survival and oviposition on this non-host plant.

Phytoplasma effectors have been shown to have an impact on plant morphology. Hoshi *et al.* found that the OY-M protein TENGU was responsible for proliferation of shoots in host plants. The appearance of these shoots lead to the name TENGU, as a mythical goblin that lives in a nest (tengus' nest) (Hoshi *et al.* 2008). Bai *et al.* described the nuclear localization signal (NLS) of AY-WB secreted protein SAP11, showing that this protein targets host cells and is potentially involved in mediating host response to phytoplasma infection (Bai *et al.* 2009).

In chapter 4 I show that AY-WB secreted protein SAP11 plays a role in suppressing the plant defence response to leafhopper feeding and oviposition (Sugio *et al.* 2011).



In chapter 5, I show that other AY-WB secreted proteins, particularly those transcribed with SAP11, have an impact on host and non-host insect-plant interactions.

This work comprehensively demonstrates that AY-WB phytoplasma utilises secreted effector proteins in the plant that change insect-plant interactions, both in known host interactions and in naïve non-host interactions. Changes caused by effector proteins in plant hosts, facilitate changes to insect vector population biology, thus, affects the epidemiology of the phytoplasma. In addition, I demonstrate that phytoplasma infection within the insect also has an effect on these interactions when insects become vectors. This indicates that titre of phytoplasmas within the insect is key to having an effect on insect-plant interactions. This research highlights the benefit of use of model systems in understanding how pathogens manipulate their hosts to increase their dispersal.

## **Materials and Methods**

### *M.1 Maintaining Macrosteles quadrilineatus*

*M. quadrilineatus* is not a native leafhopper to the UK and is an economically important pest species in North America. As such, in the UK, *M. quadrilineatus* must be maintained in strict quarantine under a scientific license. *M. quadrilineatus* were kept in controlled growth rooms in a containment facility building licensed by the Food and Environment Research Agency (FERA) under a license to import, move and keep prohibited invertebrates; PHL 185E/6284. *M. quadrilineatus* were reared and maintained in 0.52 x 0.48 x 0.52 m<sup>3</sup> clear plastic insect cages in a growth room set at 16/8-hour light/dark period at 26/20°C. The insect cages were equipped with fans for ventilation and water trays beneath in order to keep plants hydrated, without a drowning hazard to insects. Each cage housed one colony and was started with ca. 50 female and male *M. quadrilineatus* adults and 2 pots containing ca. 30 oat plants (*Avena sativa* L.). A population of ca. 1000 leafhoppers was generated in 4-6 weeks. New colonies were set up regularly with adults from existing colonies as described above. Colonies over 8 weeks old were closed down and cleaned out. This regular cycle of colony cages maintained healthy young insects and minimized risk of contamination with other insects such as aphids, or fungal or bacterial pathogens of insects. If older insects were needed for experiments, cages were kept longer and care was taken to remove honeydew from the base of the cage each week. Honeydew was removed in darkened chamber with a spotlight at the back of the cage to attract insects away from the cage door. Honeydew was wiped away using a warm damp cloth with no detergent (contact with detergent would affect insect protective wax cuticle covering, making them vulnerable to infection). After cleaning the colony cage was returned to normal light conditions. This cleaning minimized the risks of bacterial or fungal infection and the risk of insects sticking to the base of the cage.

### *M.2 Maintaining Dalbulus maidis*

*Dalbulus maidis* is not a native leafhopper to the UK and is an economically important pest species in its native South and Central America. As such, it is maintained in our secure quarantine facility under the PHL 185E/6284 license awarded by FERA. Rearing of the corn leafhopper *D. maidis* was conducted using the same methods used for rearing *M. quadrilineatus* with the exception of that host plants were in this case maize. Maize plants were grown in larger pots to benefit plant fitness, and increase plant longevity. Due to the pot size only four pots could be kept in a cage so two were added at first then more were added later in the colony life. *D. maidis* colonies took 5-7 weeks to establish a colony of approximately 1000 individuals, therefore colonies were maintained for 10 weeks. New colonies were set up frequently (as with *M. quadrilineatus*) to minimize risk of contamination and disease. If older insects were required, colony cages were maintained for longer and the bases regularly cleaned of honeydew.

### *M.3 Generating populations of leafhoppers of the same age*

To obtain leafhoppers of the same age, ca. 50 male and female leafhoppers were added to a fresh cage with 2 pots of new plants (oats for *M. quadrilineatus* and maize for *D. maidis*). The insects were left in these cages for 7 days, then all adults were carefully removed. During this time the insects will have mated and oviposited into plant tissue, but the eggs will not have hatched. The plants were left in the cages and eggs allowed to hatch and nymphs develop. The nymphs of both *M. quadrilineatus* and *D. maidis* go through 5 larval instars before their final molt into adults (eclosure). From egg hatch to eclosure took 3 weeks for *M. quadrilineatus* and 3.5 weeks for *D. maidis*. Most insects used in experiments were 2-day-old adults, thus, used two days post eclosure.

#### *M.4 Raising and maintaining plants for use in experiments*

##### *Maintaining Arabidopsis*

*Arabidopsis thaliana* ecotype Columbia-0 seeds were obtained from the John Innes Centre, Norwich, Norfolk, UK and sown onto packed and water soaked Levington F2 compost in small pots. These pots were immediately placed at 4°C for 4 days to vernalize. This cold treatment was carried out to encourage uniform germination and seedling growth (without this vernalization step, seeds exhibit variable germination and development). Pots were moved to a controlled growth room with a 10 hour day of fluorescent lighting at a constant temperature of 20°C to germinate and grow. After 10 days the seedlings were approximately 10mm tall and were transplanted, using forceps, to individual pots in trays. After a further 3 weeks growth seedlings were transplanted to larger pots and used in experiments. The cool short day conditions of 20°C and 10 hour days prolonged the rosette stage in plant development and encouraged uniformity, and delayed bolting, senescence, and death of the plants.

##### *Maintaining China Aster (Callistephus chinensis)*

Aster seeds were sown onto lightly packed M2 Levington compost in 24 well seedling trays and covered with a 2mm layer of sieved M2 compost. Seeds were allowed to germinate and grow in greenhouse conditions for 6 weeks before being potted up to larger pots. These were then grown in the same conditions for a further 3 weeks when they were used for experiments. Plants used in experiments were approximately 6 inches tall. The aster variety used was the tall growing china aster variety 'bridesmaid' from Sutton Seeds, Woodview Road, Paignton, Devon, UK.

##### *Maintaining Lettuce (Lactuca sativa)*

Lettuce varieties used were Parris island cos and Lobjoits green cos, both supplied by Ball Colegrave, West Adderbury, Banbury,

Oxfordshire, UK. Seeds were sown onto lightly packed M2 Levington compost in 24 well seedling trays and covered with a 2mm layer of sieved M2 compost. Seeds were left to germinate and grow in greenhouse conditions for 4 weeks before being potted up to larger pots. These were then grown in the same conditions for a further 3 weeks when they were used for experiments. Plants used in experiments were approximately 6 inches tall.

#### *Maintaining Maize (Zea mays)*

Maize seed variety early sunglow (*Zea mays variety rugosa*) was obtained from Park Seed Co. Greenwood, South Carolina, USA, were sown into 14cm diameter pots. Three seeds evenly spaced were pushed a depth of 3 inches into packed Levington M2 compost. Soil was distributed to cover the holes and the seeds were left to germinate and grow in greenhouse conditions for 4 weeks before being staked up to stabilize. These were then grown in the same conditions for a further 1 week when they were used for experiments or 3 weeks when used as insect food in colonies. Plants used in experiments were approximately 9 inches tall for food they were approximately 16 inches tall.

#### *Maintaining Oats (Avena sativa L.)*

Oat seed was obtained from John Innes Centre, Norwich, Norfolk, UK, and sown onto packed Levington M2 compost in 14cm diameter pots. Ca. 30 seeds evenly spaced were covered with 1cm of compost and seeds were left to germinate and grow in greenhouse conditions for 4-5 weeks when they could be used as host plants in *M. quadrilineatus* colonies. Plants used in colonies were between 6-12 inches tall.

#### *M.5 Maintaining AY-WB phytoplasma in lab conditions for experiments*

The phytoplasmas were kept under a FERA Plant Health License to import, move and keep prohibited plant pathogens. AY-WB phytoplasma was sustained within colonies of infected *M.*

*quadrilineatus* (under license detailed earlier) and infected china aster plants in plastic cages within a controlled growth room within an insect containment facility building. To generate AY-WB-infected leafhoppers, ca. 50 female and male *M. quadrilineatus* leafhoppers were transferred to AY-WB-infected China aster for two weeks to allow acquisition. Adults were removed and placed in new colony cages to inoculate new healthy china aster plants. A colony of ca. 500 infected *M. quadrilineatus* was obtained in approximately 6-7 weeks. AY-WB infected colonies were kept for 10-12 weeks, then closed and cleaned out. New cages were set up regularly with healthy china aster plants and ca. 50 AY-WB infected *M. quadrilineatus*. The recycling of colony cages minimized the risk of contamination with other insects such as the western flower thrip *Franklinella occidentalis*, to which china aster is a favored food plant.

#### *M.6 Maintaining infected insects of the same age*

AY-WB infected China aster plants were placed into colony cages with ca. 100 healthy *M. quadrilineatus* adults for 7 days. Adults were removed and eggs were allowed to hatch and develop. Newly eclosed adults raised on AY-WB infected china aster were approximately 3 weeks old giving them ample time to acquire and become vectors of AY-WB. The adults were used for experiments or were transferred to young China aster or Arabidopsis seedlings to generate new colonies of AY-WB-infected plants and leafhoppers.

#### *M.7 Analysis of AY-WB symptoms in Arabidopsis*

Arabidopsis Col-0 plants were grown in 10/14 hour light/dark at 22°C. This cool short day condition prolonged rosette stage, encouraged uniformity in plant development, and delayed bolting, senescence, and death of the plants. Trays of ten 4 week-old plants were exposed to twenty *M. quadrilineatus* leafhoppers (2 leafhoppers per plant) enclosed within perforated bags (300 x 400mm) for inoculation. A total of 20 plants were exposed to non-infected leafhoppers (referred to as

healthy plants), and another 20 plants to AY-WB-infected leafhoppers (infected plants). Following addition of insects, all plants were either returned to the short day conditions or transferred to warmer longer day conditions to assess how symptoms developed. The warmer growth room was set to 16/8 hour light/dark at 23°C/20°C. After one week, all insects were removed. Symptoms of infection, including stunted growth and yellowing of developing rosette leaves were recorded. Date of bolting, stem height and number of stems were counted, and plant senescence, deterioration and death were recorded.

#### *M.8 Measuring insect survival*

Experiments were carried out in controlled growth chambers of 10/14 hour light/dark at 22°C or 16/8 hour light/dark at 24°C/20°C or 16/8 hour light/dark at 26°C/20°C depending on the hypothesis.

Survival was recorded as the number of female *M. quadrilineatus* or *D. maidis* remaining alive on experimental plants after 4 days. Two males and 8 females from reared population cages were placed on 7 week old Arabidopsis plants (with various treatments) for 4 days. Surviving adults on these individual plants were then counted and removed. Two more males and 8 females from the initial population (now 4 days older) were placed on new 7 week old Arabidopsis plants. After 4 days, surviving adults were counted and removed. This cycle was repeated every 4 days until there were no adults left in the rearing population cage (~ 60 days old adults). The female counts were used as an explanatory variable of the insect survival. Each of the four day time periods were using independent insects and plants and therefore created a time series of independent counts of survival of the female insects over the insects age-span. Note, this method does not examine long-term survival or sub-lethal on individual leafhoppers, it provides an indication of the short-term survival of leafhoppers during experimental conditions.

#### *M.9 Measuring fecundity*

Fecundity was recorded as a count of the number of nymphs produced on each experimental plant. All experimental plants were retained in growth conditions described above for a further 15 days after adult insects were removed. At this point all live nymphs on each plant were counted. To avoid double counting a mouth aspirator was used to remove nymphs as they were counted. These nymph counts were used as a response variable for insect fecundity. This provided a measure of the viable offspring of the insects on these plants. Rather than their potential fecundity, which could be assessed by the number of eggs adult females possessed. To enable the female survival to be a factor of nymph production, these values were then used as an explanatory variable of female fecundity in the analysis.

#### *M.10 Assessing leafhopper feeding*

To examine the feeding of *M. quadrilineatus*, AY-WB infected leafhoppers and uninfected leafhoppers were enclosed on single uninfected Arabidopsis leaves for 5 days. Cages were 15ml falcon tubes with leaves (still attached to plant) gently placed inside cage and sealed with a foam bung. After 5 days, adults were removed and leaves were cut and stained with trypan blue to highlight feed/probe sites. Leaves were examined under a Nikon E800 fluorescent microscope and all feed/probe sites on central veins, secondary veins, tertiary veins and open tissue were counted. Feed/probe sites morphology was examined by randomized selection of 10 circular quadrats per leaf. The number of stylet tracks and stylet puncture points were counted for each feed/probe site. Feed/probe sites were counted separately if stylet puncture points were more than an epidermal cell width apart.

#### *M.11 Choice tests*

Single insects were added to a choice chamber cage with 3 or 4 plants randomized positions. Insects were observed for 15 minutes, their first plant visit and first settlement to feed was recorded. Plants were replaced after two insects had used the chamber and the sex of the



initial leafhopper was recorded. To assess leafhopper feeding preference additional leaf choice chambers were used. 15ml falcon tubes were cut resulting in two open ends. Arabidopsis leaves were gently placed in either end with a foam bung (still attached to plant). Five leafhoppers were enclosed for 2 days and leaves were then cut, stained with trypan blue, cleared with chloral hydrate and mounted onto slides in 80% glycerol. Feed/probe sites observed on each leaf were counted, using a Nikon E800 fluorescence microscope.

#### *M.12 Leafhopper dissections and PCR of tissue*

Leafhoppers were removed from plants, knocked out with CO<sub>2</sub> and placed individually on a drop of 0.1 M potassium phosphate buffered saline (PBS) buffer. Leafhopper heads were removed by holding the tip of the abdomen gently whilst pulling away the head with the anterior legs and proboscis. The heads containing salivary glands of 10 leafhoppers were ground over ice in 100µl insect DNA extraction buffer (below) and stored at -20°C. To collect haemolymph, the central open end of the thorax was smeared onto a microscope slide and immediately collected with 10µl droplet of insect DNA extraction buffer and pipette. Leafhopper haemolymph in extraction buffer was mixed with that of 20 leafhoppers over ice and stored at -20°C. To gain access to the leafhopper gut, the thorax was gripped gently by holding the mid legs; pulling the tip of the abdomen away pulled out the gut. The visible gut section was gently pulled free and placed in 20µl of insect DNA extraction buffer. Leafhopper guts were mixed with 10 others over ice and stored at -20°C. The ovaries were separated from the gut tissue and placed in 20µl insect DNA extraction buffer over ice, mixed with those of 10 others and stored at -20°C.

#### *M.13 DNA extraction and PCR*

DNA was extracted from *M. quadrilineatus* tissues using extraction buffer (10mM Tris-HCl at pH8, 2mM EDTA, 0.2% Triton X-100 and 100µg/mL proteinase K added just prior to use). Tissue was ground

over dry ice with a plastic pestle in 100µl of insect DNA extraction buffer. The volume was increased to 500µl by adding 400µl of extraction buffer then the mix was incubated at 56°C with occasional vortex for 30 minutes. The sample was incubated at 96°C to inactivate the proteinase, cooled to -4°C and spun in a microfuge at 15000g for 5 minutes. 1µl of DNA was used in a 20µl PCR hotstart *Taq* polymerase (Promega) reaction. Primers BR (GGAAGTCGCCTACAAAAATCC) and BF (AGGATGGAACCCTTCAATGTC) were used to amplify a 900bp genomic DNA band specific to AY-WB identified in Zhang *et al.* (2004).

#### *M.14 Experimental design and replication*

All experiments were repeated 3 times unless otherwise stated. This was done in order to assess whether the experiments would yield similar results on multiple occasions. In most survival and fecundity experiments 3 plants were used per each treatment at each time point. This arbitrary choice in number of plants was formed due to the available space in the growth chambers used to conduct experiments. Multiples of 3 plants fitted easily in water trays on shelves, which could be frequently moved around within a growth room to eliminate 'edge effects' or difference in conditions in different areas of the room. In the case of survival and fecundity trials, leafhopper age ranges were broken down into 4-day time points to measure female survival and fecundity on experimental plants over a time line. New plants and insects were used for each 4-day time point so each of the time points of data were independent of each other. Experimental plants were added to water trays randomly and trays were moved around the growth rooms at random, daily. For example, the experiment in chapter 2, figure 2.2.1.2 used 3 plant treatments at 10 time points. All plants with insects at each time point were placed in a water tray and moved around the water tray and around the growth room at random daily. The whole experiment was repeated 3 times therefore 3 repeats of 3 plants at 10 time points equate to 90 plants per treatment. There were 8 females added per experimental plant, therefore 720 females were used per

treatment. Treatment was defined as each of the insect and plant combinations i.e. healthy insects on healthy plant or healthy insects on AY-WB infected plants. For example in chapters one figure 1.2.4.2 there were 2 treatments; uninfected leafhoppers on uninfected Arabidopsis and uninfected leafhoppers on AY-WB infected Arabidopsis. All figures clearly highlight treatments used in experiments.

#### *M.15 Statistical analysis*

All the statistical analysis was completed in Genstat v.13 (International Ltd, Hemel Hempstead, UK). For all data points of insect fecundity and oviposition assay, we recorded the biological replicate, adult age, and plant treatment or line as explanatory variables, and female survival as response variables. Analysis of Deviance was used to determine differences in female survival rates, egg hatch rate, fecundity, oviposition and number of feeding sites. All explanatory variables were described as factors and modeled to assess their contribution to the deviance in leafhopper survival, egg hatch rate, fecundity, oviposition rates and number of feeding sites. Female survival data and egg hatch data were analyzed using a binomial distribution within a generalized linear model (GLM). Fecundity, oviposition and feeding data were analyzed using a GLM with a poisson link function (for count data). When fecundity and oviposition data were analyzed, female survival in each experiment was added as a co-variate. A typical model used is replicate\*plant line\*leafhopper age+female survival. Choice test data were examined using contingency tables and chi square analysis within Genstat. Error bars shown in figures are 2x standard error above and below means unless otherwise stated. All survival and fecundity figures were produced in Microsoft Excel using predicted means and standard errors imported from analysis in Genstat.

**Chapter One: AY-WB manipulation of the model plant *Arabidopsis thaliana* gives vector leafhopper increased fecundity.**

**Introduction**

*1.1.1 Phytoplasmas need vector insects to disperse in crops.*

Phytoplasmas are unculturable Gram-positive bacterial plant pathogens that belong to the Class Mollicutes. All are restricted to plant phloem sieve elements, which transport carbohydrates and nutrients around plants (Hogenhout *et al.* 2008). Phytoplasmas rely on phloem-feeding Hemiptera as insect vectors, usually leafhoppers, planthoppers and psyllids, for transmission to plants (Weintraub and Beanland 2006). Therefore, phytoplasmas are obligate pathogens of plants and insects depending on both hosts for dispersal. Phytoplasma dispersal is limited to the host range of its vector insects (Beanland *et al.* 2000).

*1.1.2 Macrosteles quadrilineatus is a prolific vector of Aster yellows phytoplasmas*

Aster Yellows phytoplasmas (AYPs) collectively have a wide plant host range affecting more than 80 dicot and monocot plant species worldwide (Hogenhout *et al.* 2008). These include many economically important crop and ornamental species (O'mara *et al.* 1993). In the United States of America and Canada, AY phytoplasmas are primarily vectored by *Macrosteles quadrilineatus*, which has an equally wide plant host range (Wilson and Turner, 2010). *M. quadrilineatus* is migratory and its range determines the transmission of AYPs to crops on route. Normal infective individuals make up 4-5% of *M. quadrilineatus* populations but this can rise in some years to over 10%, which can cause significant crop yield losses in a season (O'mara *et al.* 1993). As such, *M. quadrilineatus* and AYPs are monitored by many State agriculture departments, scored and guides put in place for growers (Chaput and Sears, OMAFRA, 1998). These data are inevitably more

pertinent to the protection of crops in northern leafhopper ranges as growers have time to act on information received from Southern States (O'mara *et al.* 1993). *M. quadrilineatus* has a high risk of migrating to new regions especially when human activity facilitates movement. In Hawaii, for example some AYP infected *M. severini* or *M. quadrilineatus* individuals have caused great yield losses in watercress since arriving on cargo from Southern USA (Hue *et al.* 2003; Borth *et al.* 2006). The impacts of *M. quadrilineatus* and AYPs are compounded by numbers of leafhopper sibling species within the quadrilineatus-complex and mixed AYP isolates within leafhoppers (Kwon 1988; Borth *et al.* 2006; Smith *et al.* 2002; Roux *et al.* 2008).

#### 1.1.3 *Arabidopsis* provides a powerful study tool in phytoplasma interactions

Many AYPs can infect many plants of the family Brassicaceae (O'mara *et al.* 1993), the majority of which are economically important crops such as *Brassica oleracea* varieties: cauliflower, broccoli, and cabbage. Many weed brassica species, found surrounding crop fields serve as reservoir plants for AYPs such as *Capsella bursa-pastoris* (Shepherd's purse) (O'mara *et al.* 1993; USDA plant database. 2011; Munyaneza *et al.* 2006). Interestingly, *Capsella bursa-pastoris* shares comparable ecology and gene expression during its life cycle to the model Brassicaceae *Arabidopsis thaliana* (Mouse-ear cress) (Yang *et al.* 1998; Slotte *et al.* 2007). *Arabidopsis thaliana* ecotypes are found worldwide in similar environments to *Capsella bursa-pastoris* (USDA plant database. 2011; Tair. 2011). In USA and Canada *Arabidopsis thaliana* is found in the majority of States where AYPs and *M. quadrilineatus* are present (Fig. 1.1.3.1) (Clarke. 1993; Hoy *et al.* 2003; Hoy *et al.* 2006). If *M. quadrilineatus* and AYPs can utilise *Arabidopsis* as a host, researchers would have a huge genetic resource with which to investigate phytoplasma modulation and pathogenicity in plant hosts.



**Figure 1.1.3.1. Many States with *Arabidopsis* are visited by migrating *M. quadrilineatus***

*Arabidopsis thaliana* ecotypes are found in all States highlighted in green where in many cases *A. thaliana* is listed as a noxious weed to crops (data compiled from USDA plant database 2011 and Clarke 1993). *M. quadrilineatus* migrations are highly influenced by wind and weather systems and as such vary from year to year. In general, *M. quadrilineatus* are found in Texas, Louisiana, Arizona and Oklahoma in spring and migrate east to New Jersey, Maryland, and New York; north-east to Ohio, Michigan, and South Ontario (Canada); north to Minnesota, North Dakota, and South Ontario (Canada); north-west to Oregon and Montana and west to California (data compiled from Chiykowski and Chapman 1968; Hoy *et al.* 1992; Hoy *et al.* 1999; Kwon 1988; O'Mara *et al.* 1993; Olivier *et al.* 2010 and OAMFRA pest database). Map taken from Google maps, scale bar is 1000 miles.

#### 1.1.4 Assessment of *Arabidopsis* as a model system for AYP research

Aster yellows phytoplasma strain witches' broom (AY-WB) was originally isolated from a mix of AYPs in infected lettuce in Ohio by Zhang *et al.* 2004. In 2006 that phytoplasma isolate was fully sequenced and now AY-WB is one of four fully sequenced phytoplasmas (Bai *et al.* 2006; Bai *et al.* 2009). In this chapter I demonstrate this fully sequenced phytoplasma is efficiently vectored by *M. quadrilineatus* into *Arabidopsis thaliana* ecotype Columbia (Col-0). *Arabidopsis* Col-0 is found with other weeds in approximately 50% of Ohio State, where this phytoplasma is present (USDA plant database; Furlow 1991; Weishaupt 1971). In addition, I show that AY-WB infection affects morphology of

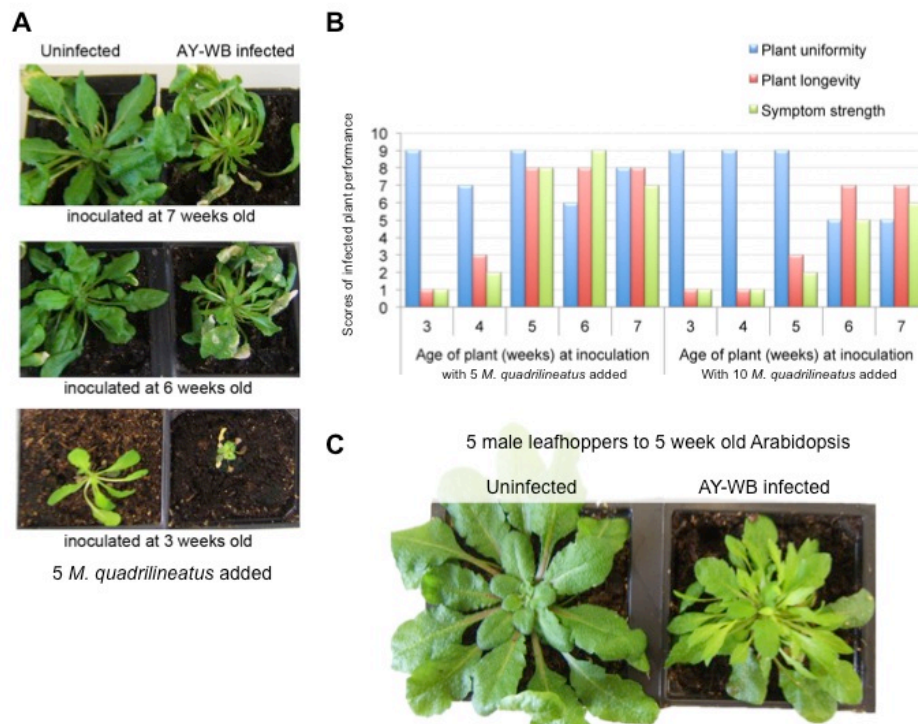
*Arabidopsis* Col-0. Furthermore, I demonstrate that AY-WB infection in *Arabidopsis* Col-0 renders the plant more suitable for oviposition of *M. quadrilineatus*. These changes in plant morphology and susceptibility to vector oviposition highlight that *Arabidopsis thaliana* in Ohio is potentially a reservoir host in nature. In addition, these results advocate *Arabidopsis* model system use in AYP research. Use of this model system opens new avenues of research into pathogenicity and manipulation of host plants by AYPs.

## Results

### 1.2.1 AY-WB has increased pathogenicity in young *Arabidopsis*

Phytoplasma infection of *Arabidopsis* was initially confirmed by exposing plants to AY-WB infected male *M. quadrilineatus* for one week and following removal, plants were monitored for 3 weeks and assayed for AY-WB using PCR. During this infection process, I observed substantial variation in plant fitness, longevity and symptom development. To properly examine the alterations caused by phytoplasma infection in plants I needed to find plant infection conditions that presented minimal variability, strong symptoms and good longevity of plants so the plants could endure the experiments. To determine an optimal plant age to yield uniform infected plants for experiments, I conducted an infection screen of *Arabidopsis* at various ages. *Arabidopsis* aged 3, 4, 5, 6, and 7 weeks were inoculated with either 5 or 10 leafhoppers for one week and symptoms were recorded for a further 4 weeks. *Arabidopsis* aged 3, 4, or 5 weeks had poor survival with inoculations of 10 insects (Fig. 1.2.1.1 A and B). Three-week-old plants did not survive long enough to develop strong AY-WB symptoms. Screened plants were scored by longevity, strength of symptoms and uniformity of development. *Arabidopsis* that were inoculated with 5 leafhoppers when plants were 5 weeks old had the

highest score, with good survival time and the most uniform symptoms (Fig. 1.2.1.1B and C).



**Figure. 1.2.1.1. Arabidopsis inoculated at 5 weeks by 5 male leafhoppers exhibit uniform symptoms.**

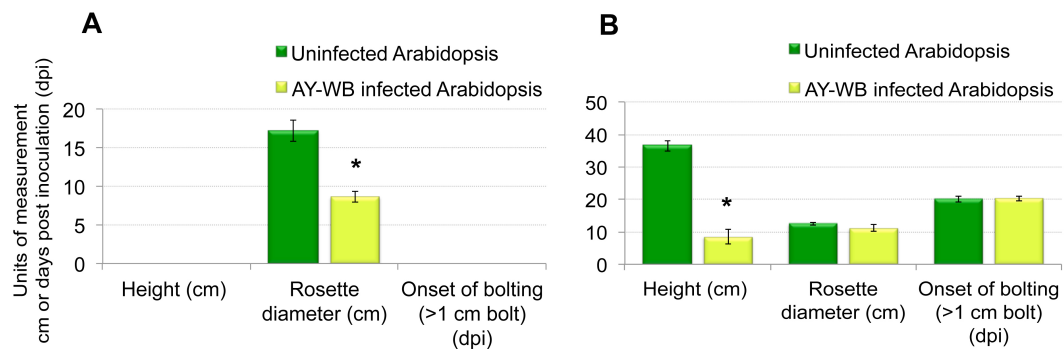
Uniformity in experimental plants was an important target to minimize noise in data when looking at how phytoplasma infection alters plant-insect interactions. Plant uniformity, longevity and symptom development were visually scored out of 10 when observing trays of 12 plants after inoculations in different conditions. Arabidopsis plants were inoculated between 3 to 7 weeks in age using 5 or 10 male *M. quadrilineatus* and symptoms were left to develop. Plants inoculated by 5 leafhoppers had better longevity than those inoculated by 10 leafhoppers (B). Uniformity is high in inoculations at 3 weeks due to most of these plants dying within 2 weeks (B) although a few plants did survive (C). Arabidopsis inoculated at 5 weeks by 5 leafhoppers had consistently high scores for symptoms, longevity and uniformity (B and C). Thus this condition was deemed the most suitable condition to treat infected plants for experiments. Whole experiment repeated twice.

### 1.2.2 AY-WB induces stunting and leaf proliferation in Arabidopsis

In order to quantify morphology changes to Arabidopsis as a result of AY-WB infection, symptom development was recorded every 2 days for 4 weeks. Myself, and lab colleague, Dr Allyson MacLean, completed this



screen. Plants in this screen were 4 or 5 weeks old, inoculated with uninfected or AY-WB infected male *M. quadrilineatus* and kept in short or long day conditions. Controlled growth rooms were used to create short and long day conditions; room A was set to cool, short day conditions at 22°C consistently with a 10 hour day and 14 hour night. Room B was set for warmer, longer day conditions at 26°C 16 hour day and 22°C 8 hour night. We found plant height and rosette diameter was significantly reduced on AY-WB infected Arabidopsis (Fig. 1.2.2.1 B). AY-WB infected Arabidopsis flowered later than uninfected plants, and flowers in long day conditions were green leafy flowers (Fig. 1.2.2.1 B). Rosette diameter was significantly reduced in AY-WB infected Arabidopsis when plants were grown in short day conditions at 22°C consistently with a 10 hour day and 14 hour night (Fig. 1.2.2.1 A).



**Figure. 1.2.2.1. AY-WB infected Arabidopsis are smaller than uninfected Arabidopsis.**

Arabidopsis plants were inoculated at 4 weeks and 5 weeks after sowing. Two controlled growth rooms were used, room A was set to cool conditions at 22°C consistently with a 10 hour day and 14 hour night. Room B was set for warmer conditions at 26°C 16 hour day and 22°C 8 hour night. Ten plants per treatment per room were measured for growth. Plant growth was measured over 44 or 54 days post inoculation (dpi) for A and B conditions, respectively. AY-WB infected plants were significantly shorter than uninfected plants ( $P = <0.001$ ). AY-WB rosette diameter was also significantly reduced in room A and B conditions ( $P = <0.001$  [A] and  $P = 0.05$  [B]). Data analysed using Student's t-tests. (In room A no plants bolted within 44 dpi. Height was measured on plants with >1cm bolts).



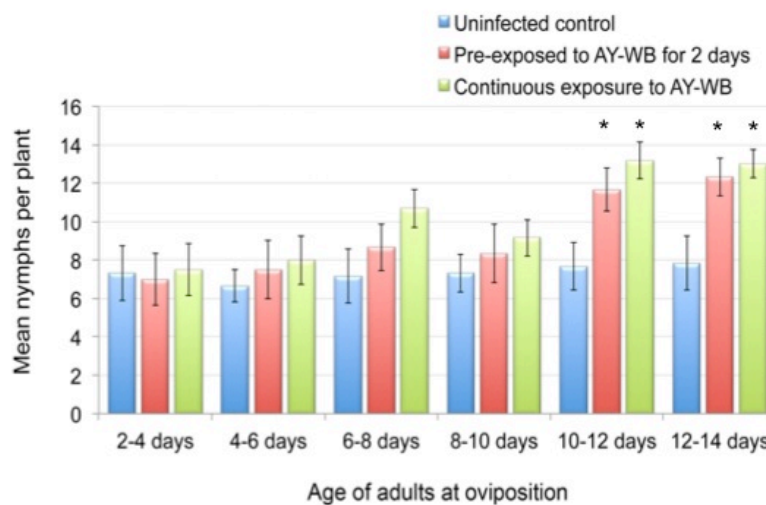
**Figure. 1.2.2.2. Arabidopsis flowers develop as leafy structures when infected with AY-WB**

Arabidopsis flowers are frequently green, leafy and covered in trichomes when plants are infected with AY-WB before the plant has bolted. Petals become leaves and stamens are stunted and malformed. This plant was inoculated after 5 weeks growth and the photo was taken after 9 weeks growth. Photograph taken by Andrew Davis, John Innes Centre, Norwich, UK.

### *1.2.3 AY-WB impact on infected leafhopper fecundity only occurs at least 10 days post infection*

To separate effects of phytoplasma infection in the plant and the insect in insect-plant interactions, a time series experiment needed to be designed. A times series approach would give insight as the point AY-WB infection within the insect has an impact on insect-plant interactions. This would allow future survival and fecundity trials to be carried out with minimal blurring of the origin of the effects. To assess at which point after infection AY-WB within the insect has an effect on fecundity, I conducted a survival and fecundity assay over the infection period. Leafhoppers were raised on uninfected Arabidopsis and subsequently either remained on uninfected Arabidopsis or were exposed to AY-WB infected Arabidopsis just 2 days prior to transfer to

the experimental plants. Experimental plants were AY-WB infected or uninfected Arabidopsis. Leafhoppers were added to new plants every 2 days and surviving adults were counted. After a further 15 days nymphs were counted on experimental plants. *M. quadrilineatus* exposed to AY-WB-infected Arabidopsis continuously did not show an increase in fecundity for up to 6 days (Fig. 1.2.3.1). *M. quadrilineatus* exposed to AY-WB infected Arabidopsis for just 2 days prior to transfer to uninfected Arabidopsis has increased nymph production after 10 days (Fig. 1.2.3.1). These data indicate that AY-WB infection in the insect increases fecundity only after incubation period of 10 days. There is an insignificant increase in fecundity after 6-8 days incubation. Therefore, future experiments should include time points of exposure of less than 6 days, to minimize effects of infection in both hosts.



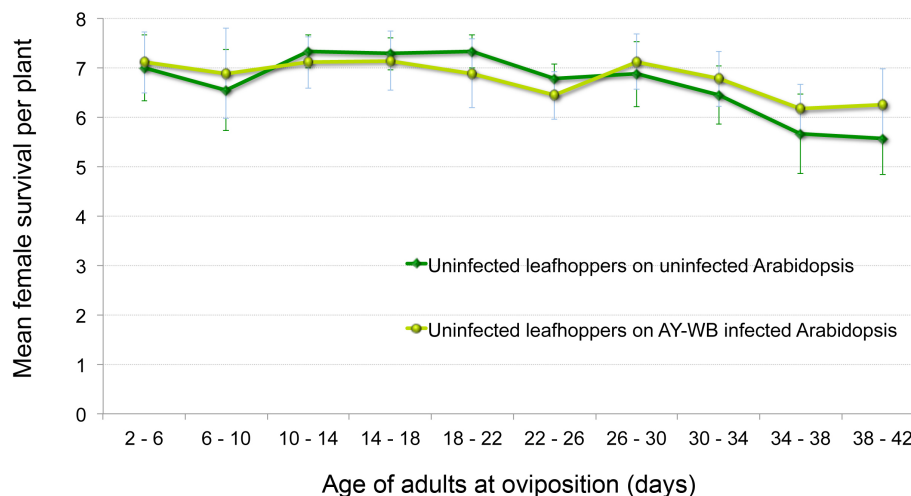
**Figure. 1.2.3.1. AY-WB *M. quadrilineatus* fecundity is increased 10 days after exposure to AY-WB**

Leafhoppers were raised on uninfected Arabidopsis until adulthood. At eclosure insects were placed on new experimental plants every two days. *M. quadrilineatus* went through three different successions of experimental plants; uninfected Arabidopsis every 2 days, AY-WB infected Arabidopsis for initial 2 days then uninfected Arabidopsis every 2 days, or AY-WB infected Arabidopsis every 2 days throughout the experiment. All plants were retained after exposure to insects for 15 days, at which point hatched nymphs were counted. Each experiment included 3 plants per age range per treatment and the experiments were repeated twice. The fecundity of insects exposed to continuous AY-WB infected Arabidopsis and only for 2

days was significantly higher after 10 and 12 days than uninfected controls \*  $F_{2,36} = 2.87$ ,  $P < 0.001$ . Exposure to AY-WB increased fecundity significantly over the experiment  $F_{2,84} = 10.68$ ,  $P < 0.001$ . Each experiment included three plants per treatment per time point were used and the experiment was repeated twice. Modeled by rep\*treatment\*age. Error bars shown are 2x standard error above and below means.

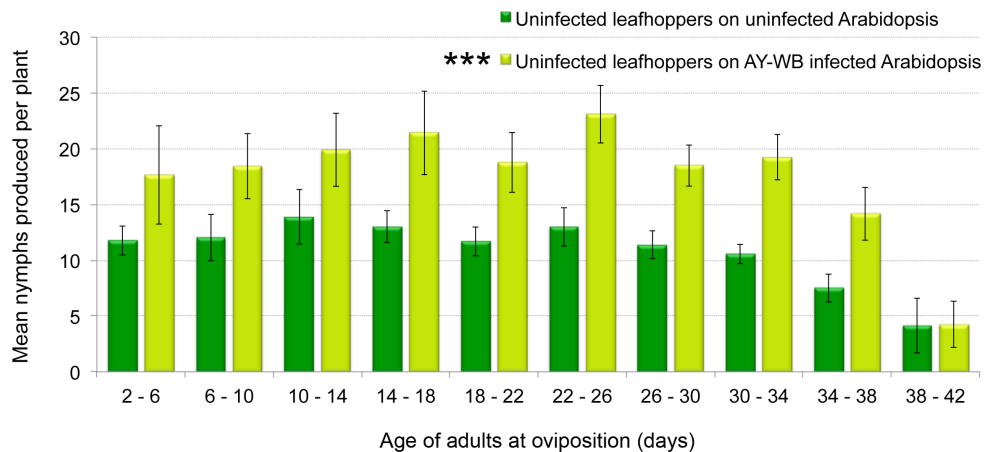
#### 1.2.4 *M. quadrilineatus* has increased fecundity on AY-WB infected *Arabidopsis*.

To determine whether AY-WB in *Arabidopsis* has an effect on the fitness of its vector *M. quadrilineatus*, I tested leafhopper survival and fecundity on individually enclosed *Arabidopsis*. Plants were pre-inoculated with either uninfected or AY-WB infected male *M. quadrilineatus*. Survival was recorded after 4 days of enclosure on plants and live adults were removed. High survival was recorded on all test plants and no significant difference in fitness in terms of survival was observed (Fig. 1.2.4.1). Emerging nymphs were counted 15 days post-adult removal, as a measure of fecundity. The fecundity of *M. quadrilineatus* on infected *Arabidopsis* was significantly higher than on uninfected *Arabidopsis* (Fig. 1.2.4.2). This increase in fecundity indicates that AY-WB infection in *Arabidopsis* does affect leafhopper fitness in terms of reproduction but not longevity.



**Figure. 1.2.4.1. *M. quadrilineatus* survival is unaltered AY-WB infection**

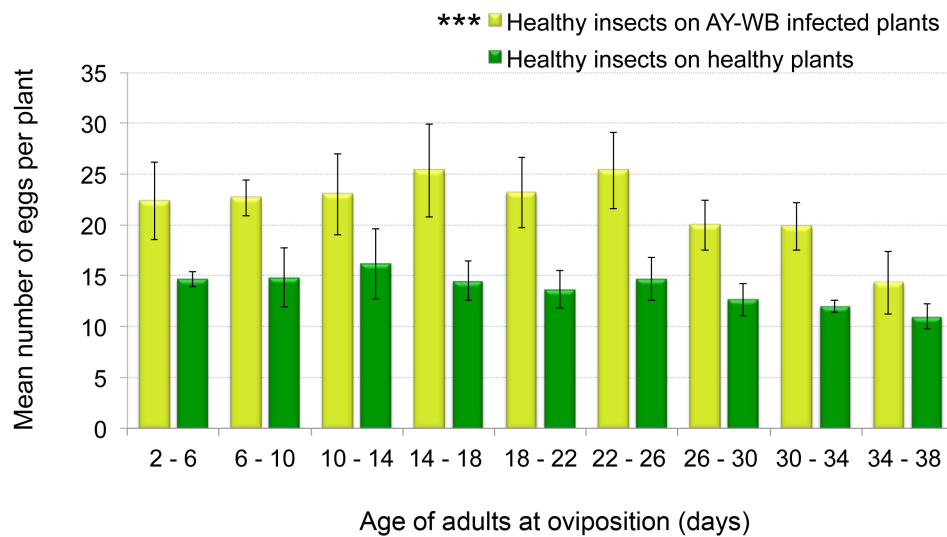
Uninfected leafhoppers were enclosed for 4 days on AY-WB infected Arabidopsis or uninfected inoculated Arabidopsis and male and female survival was recorded. *M. quadrilineatus* has high survival on Arabidopsis over 4 days and there was no significant difference seen between treatments  $F_{1,166} = 0.226$ ,  $P = 0.602$ . Each experiment included 3 plants per age range per treatment and the experiments were repeated three times. Modeled by rep\*treatment\*age using GLM with a binomial link function. Error bars shown are 2x standard error above and below means.



**Figure. 1.2.4.2. *M. quadrilineatus* has increased fecundity on AY-WB infected Arabidopsis.** Uninfected *M. quadrilineatus* leafhoppers produce more nymphs on AY-WB-infected plants,  $***F_{1,106} = 254.17$ ,  $P < 0.001$ . AY-WB-infected and uninfected Arabidopsis were obtained by exposing 5 week-old plants to three AY-WB infected and non-infected male *M. quadrilineatus*, respectively, for one week. Two weeks later 8 adult females and 2 adult males of different age ranges for 4 days (x-axes) were added to experimental plants. Nymphs were counted 15 days after removal of the adult insects. Each experiment included 3 plants per age range per treatment and the experiments were repeated three times. Modeled by rep\*treatment\*age+female survival using GLM with a poisson link function. Error bars shown are 2x standard error above and below means.

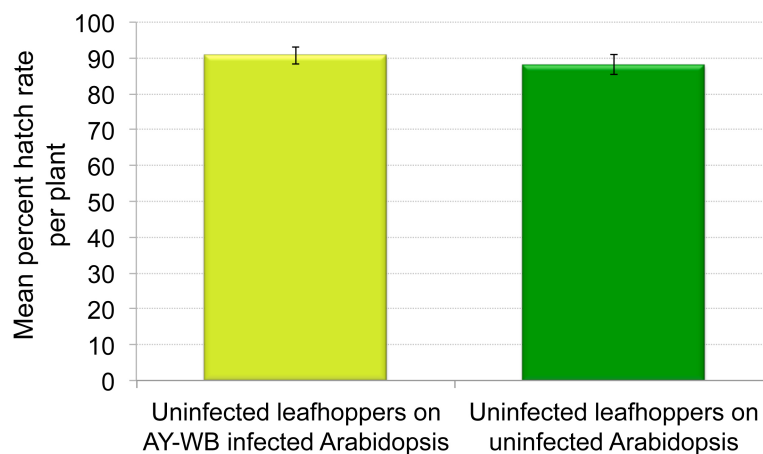
I have demonstrated that fecundity of *M. quadrilineatus* is increased on AY-WB infected Arabidopsis (in three different conditions). In order to assess whether oviposition level or hatch rate is responsible for this increase, I recorded the number of eggs per plant and used this to calculate egg hatch rates (eggs÷nymphs). *M. quadrilineatus* oviposited

more eggs on AY-WB infected Arabidopsis and hatch rate was not significantly altered between plants (Fig. 1.2.4.3 and Fig. 1.2.4.4).



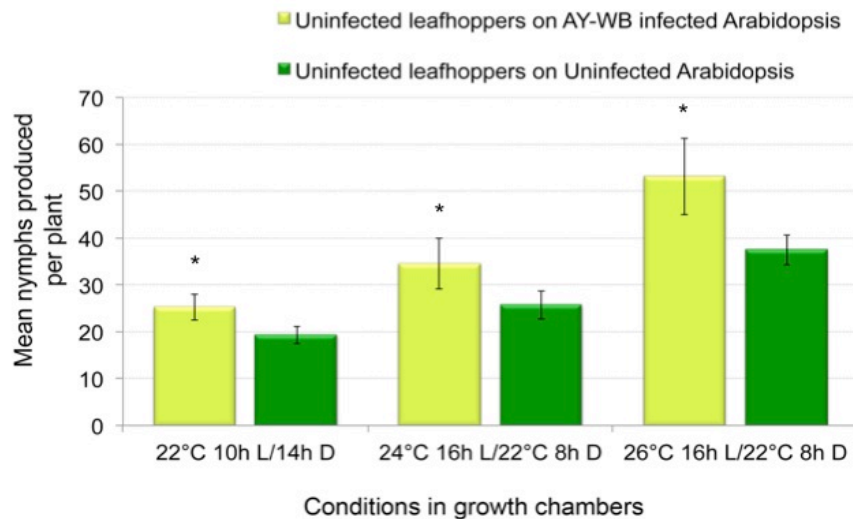
**Figure. 1.2.4.3. *M. quadrilineatus* has increased fecundity on AY-WB infected Arabidopsis**

*M. quadrilineatus* oviposit more on AY-WB-infected plants,  $***F_{1, 68} = 372.06$ ,  $P < 0.001$ . Numbers of eggs were counted one day after removal of the adult insects. Each experiment included 3 plants per age range per treatment and the experiments were repeated twice. Modeled by  $\text{rep} * \text{treatment} * \text{age} + \text{female survival}$  using GLM with a poisson link function. Error bars shown are 2x standard error above and below means.



**Figure. 1.2.4.4. *M. quadrilineatus* egg hatch rate is unchanged on AY-WB infected Arabidopsis.** Leafhoppers hatch rate was calculated by dividing the number of eggs by the number of nymphs hatching out after 15 days. Each experiment included 3 plants per age range per treatment and the experiments were repeated twice. The hatch rate on AY-WB infected Arabidopsis was not significantly different from that of uninfected Arabidopsis,  $F_{1,68} = 1.97$ ,  $P = 0.165$ . Modeled by rep\*treatment\*age using GLM with a binomial link function. Error bars shown are 2x standard error above and below means.

To examine leafhopper fitness in environmental conditions likely to be encountered by *M. quadrilineatus* migrations, survival and fecundity experiments were repeated in 3 different conditions. Controlled growth rooms were used, room A was set to cool conditions at 22°C consistently with a 10 hour day and 14 hour night. Room B was set for warmer conditions at 24°C 16 hour day and 22°C 8 hour night and room C was warmer still with 26°C 16 hour day and 22°C 8 hour night. 14-18 day-old leafhoppers were used on experimental AY-WB infected or uninfected Arabidopsis plants. I found that *M. quadrilineatus* had high survival in all conditions as in previous experiments. Fecundity was increased on AY-WB infected Arabidopsis in all conditions. Furthermore, fecundity increased significantly in each room with increase in temperature and day length, on both uninfected and AY-WB infected Arabidopsis (Fig. 1.2.4.5). In addition the variability in fecundity also increased in warmer rooms with longer days. These data indicate that in natural conditions AY-WB infection in Arabidopsis may have a more pronounced increasing effect on fecundity and that this effect may be more variable.



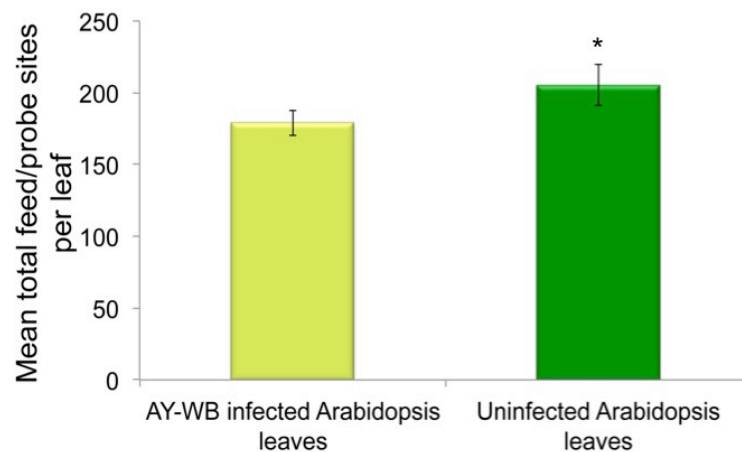
**Figure. 1.2.4.5. *M. quadrilineatus* increased fecundity is extended in warmer conditions** Leafhopper fecundity is higher in warmer rooms with longer day light on all experimental plants  $F_{2,22} = 47.55$ ,  $P < 0.001$ . Nymph numbers on AY-WB infected Arabidopsis is consistently higher than on uninfected Arabidopsis  $*F_{1,22} = 28.15$ ,  $P < 0.001$ . Error bars highlight that variability in fecundity is more pronounced in warmer rooms with longer days. Insect fecundity experiments included 3 plants per treatment per room and the whole experiment was repeated twice. Modeled by  $\text{rep} \times \text{treatment} \times \text{age}$  using GLM with a poisson link function. Error bars shown are 2x standard error above and below means.

#### 1.2.5 *M. quadrilineatus* feeding is unchanged on AY-WB infected Arabidopsis.

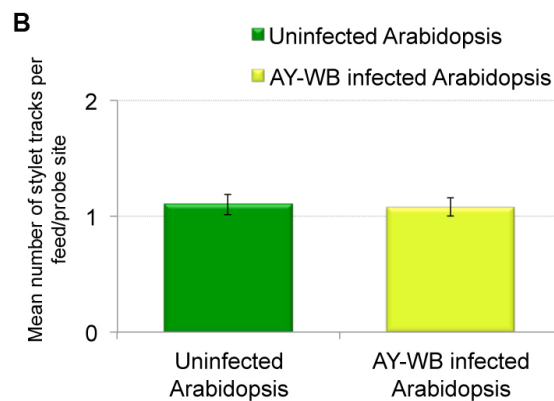
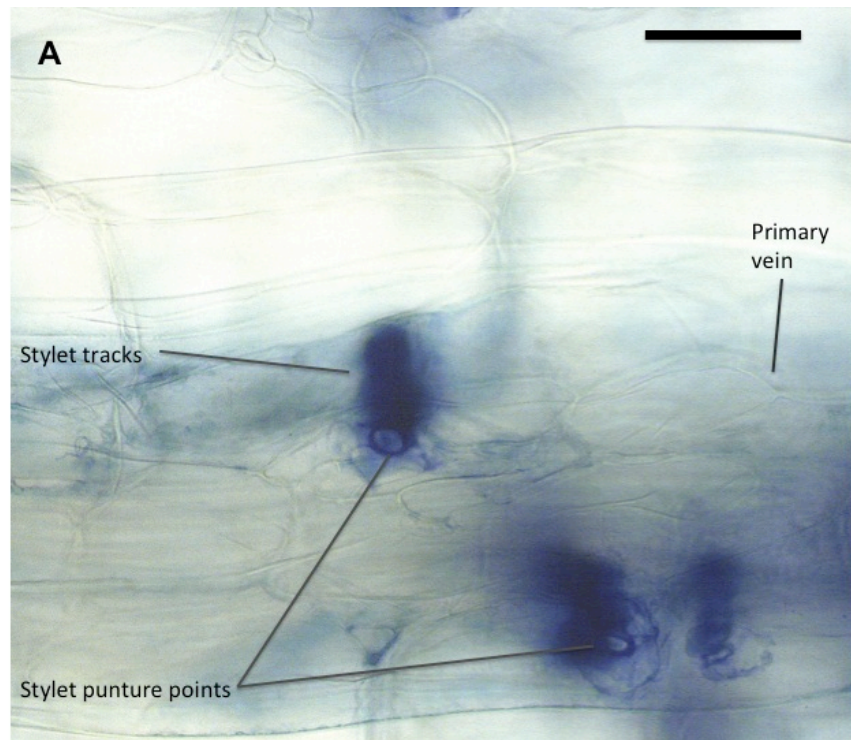
To assess the contribution of feeding levels to the increase in leafhopper fecundity, feeding sites were recorded on AY-WB and uninfected Arabidopsis leaves. Leafhoppers were enclosed on single leaves for 5 days after which leaves were cut, stained with trypan blue to highlight feeding sites, and mounted on slides. Significantly more feed/probe sites were found on uninfected over AY-WB infected leaves (Fig. 1.2.5.1). There was no significant difference in the number of stylet tracks per puncture point at feed/probe sites on AY-WB infected leaves and uninfected leaves (Fig. 1.2.5.2). Location of feed/probe sites on leaves was insignificantly different (Tab. 1.2.5.3). These results indicate that whilst AY-WB infection in Arabidopsis has limited effect on vector feeding, increase in insect fitness on AY-WB infected Arabidopsis is due



to significantly increased oviposition on AY-WB infected Arabidopsis. However to fully understand the impact of increased feeding on uninfected Arabidopsis over AY-WB infected Arabidopsis, electro penetration graphs would be a useful quantification of the feeding (Backus *et al.* 2007).



**Figure. 1.2.5.1. *M. quadrilineatus* feed/probe less AY-WB infected leaves than uninfected leaves.** *M. quadrilineatus* were enclosed on AY-WB infected and uninfected single leaves for 5 days and removed. Leaves were cut and stained with trypan to render feed/probe sites marked in blue. Total feed/probe sites were counted per leaf and their location recorded. Significantly more feeding sites were recorded on uninfected leaves over AY-WB infected leaves,  $*F_{1,22} = 32.691$ ,  $P = 0.01$ . Modeled by rep\*treatment using GLM with a poisson link function. Error bars shown are 2x standard error above and below means.



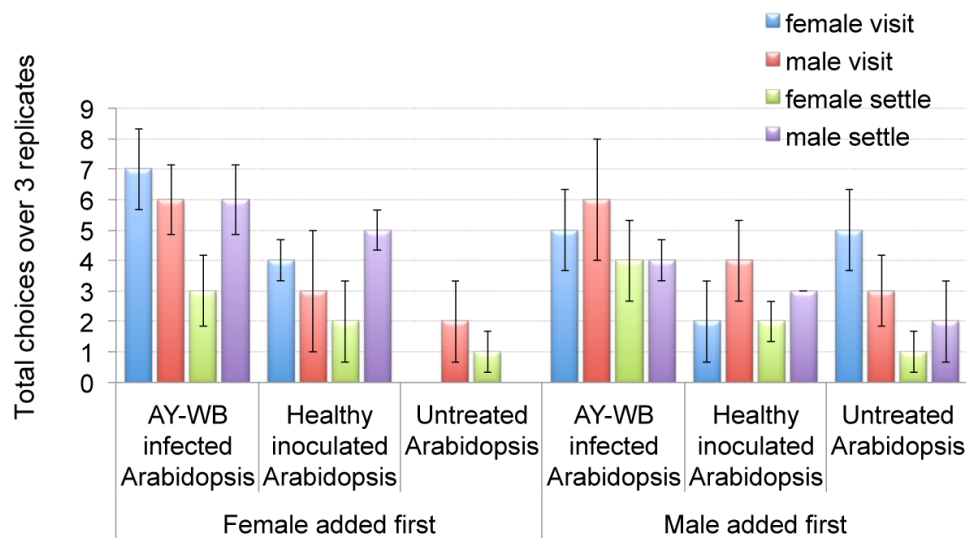
**Figure. 1.2.5.2. AY-WB in Arabidopsis makes no difference to puncture points and stylet tracks of *M. quadrilineatus* feed/probe sites.** *M. quadrilineatus* were enclosed on AY-WB infected and uninfected single leaves for 5 days and removed. Leaves were cut and stained with trypan to render feed/probe sites marked in blue. Feed/probe sites were observed under a Nikon E800 Fluorescent microscope and puncture points and stylet tracks per feeding sites were recorded in 10 quadrats per leaf. **A** The majority of feed/probe sites consisted of only one puncture point and one feeding track towards phloem. **B** No significant difference in number of stylet tracks per feed/probe site was observed on AY-WB infected leaves and uninfected leaves,  $F_{1,92} = 0.0045$ ,  $P = 0.706$ . The experiment included **A** Bar = 10 microns, **B** Modeled by rep\*treatment using GLM with a poisson link function. Error bars shown are 2x standard error above and below means.

Leaf Treatment	Feeding site location				Total
	Primary vein	Secondary veins	Tertiary veins	Open Tissue	
AY-WB infected	97.58	41.75	26.00	13.75	179.08
SE	+/-2.27	+/-2.30	+/-1.58	+/-1.51	+/-2.54
Uninfected	118.42	43.17	26.75	16.92	205.25
SE	+/-2.90	+/-2.22	+/-2.25	+/-1.17	+/-4.13

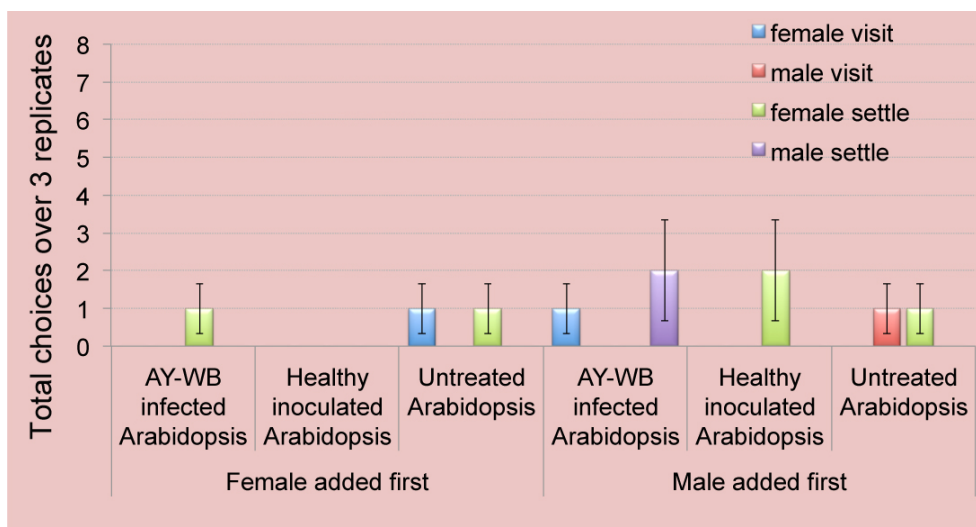
**Table 1.2.5.3. *M. quadrilineatus* feed/probe similarly on AY-WB and uninfected leaves.** Leaves were exposed to leafhoppers and stained with trypan blue to highlight feed/probe sites were examined under a Nikon E800 fluorescent microscope. Feed/probe sites were counted on primary, secondary, tertiary veins, and open tissue of leaves. *M. quadrilineatus* concentrated feeding on the primary veins  $F_{4,16} = 2.876$   $P = <0.001$ . No significant difference was observed in feed/probe site locations between AY-WB infected leaves and uninfected leaves  $F_{1,24} = 6.573$   $P = 0.283$ . Data were analysed using analysis of deviance in Genstat v.13. The experiment included 12 infected and 12 uninfected leaves exposed to 5 leafhoppers for 5 days.

#### 1.2.6 *M. quadrilineatus* has increased preference for AY-WB infected *Arabidopsis*.

To determine whether *M. quadrilineatus* has a preference for AY-WB infected over uninfected *Arabidopsis* I conducted choice chamber tests. Leafhoppers were given access to an AY-WB infected *Arabidopsis*, an untreated *Arabidopsis*, and an uninfected inoculated *Arabidopsis* in a choice chamber. The choice tests were completed with red light and with white light to test whether choice was affected by visual cues (white light) or olfactory cues (red light – leafhoppers are unable to see in red light). I found that there was no significant preference in males or females in first visit or feeding in red light (Fig. 1.2.6.2). This indicates that AY-WB infection does not alter olfactory stimuli in *Arabidopsis* to make them more attractive to *M. quadrilineatus*. I found that in white light, in which leafhoppers are able to see, male and female leafhoppers visited and settled to feed/probe on AY-WB infected *Arabidopsis* significantly more than uninfected *Arabidopsis* (Fig. 1.2.6.1). This indicates that AY-WB infected *Arabidopsis* have greater apparency or are more visually attractive to *M. quadrilineatus*.



**Figure 1.2.6.1. *M. quadrilineatus* shows preference AY-WB infected Arabidopsis in white light.** A choice chamber was used to assess whether AY-WB infected Arabidopsis are more attractive than uninfected plant. Males and females showed slight but significant preference AY-WB infected plants  $P = 0.05$ . The sex of the first adult in the chamber made no significant impact  $P = 0.14$ . In addition, if the leafhopper visited a plant it usually also settled to feed/probe  $P = <0.001$ . Data analysed using contingency tables and Pearson chi square analysis in Genstat v.13. The experiment included 6 male and 6 female leafhoppers individually tested in choice chambers for preference on AY-WB infected, healthy inoculated, and untreated Arabidopsis. Plants were replaced in choice chambers 6 times over the experiment. The whole experiment was repeated 3 times and means for each calculated and shown. Error bars shown are 2x standard error above and below means.



**Figure 1.2.6.2. *M. quadrilineatus* shows no significance response to AY-WB infected Arabidopsis olfactory stimuli.** Choice chamber experiments were repeated in red light in which leafhoppers are blind and must rely on olfactory rather than visual stimuli. Single leafhoppers were placed in the center of choice chambers as before and their movement towards plants observed for 15 mins. In most cases leafhoppers remained in the center of the chamber grooming for the entire experiment. Leafhoppers that did visit or settle on plants, did so randomly, exhibited no significant preference in visiting or settling on any Arabidopsis  $P = >0.08$  in all cases. Data analysed using contingency tables and Pearson chi square analysis in Genstat v.13. The experiment included 6 male and 6 female leafhoppers individually tested in choice chambers for preference on AY-WB infected, healthy inoculated, and untreated Arabidopsis. Plants were replaced in choice chambers 6 times over the experiment. The whole experiment was repeated 3 times and means for each calculated and shown. Error bars shown are 2x standard error above and below means.

## Discussion

### 1.3.1 *Phytoplasma impact on a model system opens new avenues of research*

*Arabidopsis thaliana* is not only a susceptible host to both *M. quadrilineatus* and AY-WB phytoplasmas. It is possible to gain multiple relatively uniform growth and symptom development in AY-WB infected *Arabidopsis* plants. This allows a solid platform on which experiments can be designed to fully qualify how phytoplasmas modulate their plant hosts.

*M. quadrilineatus* is more successful on AY-WB infected *Arabidopsis*, producing more progeny than on healthy plants. This supports findings on other plants such as Aster and Lettuce (Beanland et al. 2000; Ebbert and Nault 1998). It is hypothesized that AYPs alter infected plant defence or changes in morphology renders plants more attractive to insect, thus leading to higher levels of feeding and oviposition.

AY-WB Phytoplasma modulation of *Arabidopsis* and its interaction with *M. quadrilineatus* opens many new avenues of phytoplasma research.

The complete genome and extensive knowledge of protein functions and interactions allows the examination of specific defence pathways and plant developmental processes that may be modulated by AY-WB. Using Arabidopsis with fully sequenced AY-WB phytoplasma and the efficient vector *M. quadrilineatus* will yield valuable clues as to how AYPs modulate their plant hosts to increase their dissemination.

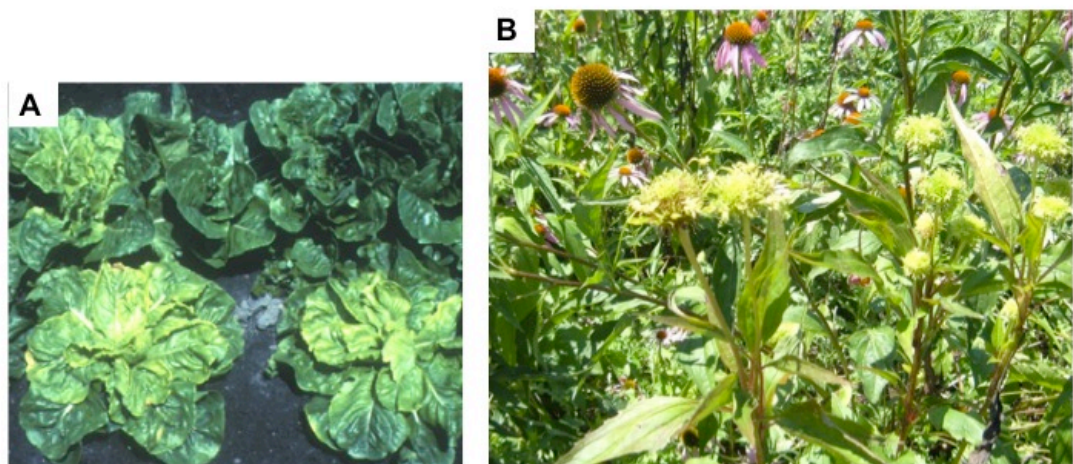
### 1.3.2 Morphological changes in plants increase apparency

AY-WB phytoplasma induces uniform symptoms in Arabidopsis thaliana if plants are grown and inoculated under specific lab conditions. Whilst this approach is removed from plants infected in field situations it does give a stable experimental set of plant on which to study phytoplasma interactions. AY-WB infected Arabidopsis are smaller lighter in colour and have proliferation of rosette leaves in comparison to uninfected Arabidopsis. It is plausible that these changes in plant morphology increase the apparency of these plants to herbivorous insects. Plant apparency is defined as form, density, longevity and persistence and these have an effect on plant defence strategy (Feeny 1976). Apparent plants largely defend themselves with low nutritional value as a consequence of visits by adapted and non-adapted insects (Feeny 1976; Futuyma 1976). Non-apparent plants are more likely to develop co-evolution arms races with specialized herbivores that use plant alleochemicals as location cues (Wasserman 1979). Changes in plant structure brought about by AYP infection can increase plant apparency (Fig. 1.3.2.1). AY-WB alters plant height, colour, leaf proliferation and greening of flowers all of which may render infected plants more obvious or attractive to herbivorous insects.

*M. quadrilineatus* visit significantly more AY-WB infected Arabidopsis than uninfected plants (Fig. 1.2.6.1). Indicating that changes in morphology induced by AY-WB may enhance plant appearance or apparency to leafhoppers. In addition, in visually impaired conditions *M. quadrilineatus* largely remains sedentary and doesn't choose a host

plant. This supports an assumption that the insect is predominantly influenced by visual cues in host plant location. This is plausible as leafhoppers including *M. quadilineatus* have been shown to be attracted to yellow light and choose to feed on young tissue of plants (Todd *et al.* 1999). These experiments would need to be repeated at different times of day and in different lights to gain a better understanding of how visual cues influence leafhopper host selection.

The use of *Arabidopsis thaliana* ecotype Col-0 is limited in use to examine volatile cues, as it has compromised volatile production due to a mutation. A potentially more suitable *Arabidopsis thaliana* ecotype would be Landsberg (Ler-1) as this has normal volatile production. Further experiments are needed to fully understand the role of phytoplasma modulation of plant morphology in changing host selection.



**Figure. 1.3.2.1. Aster yellows phytoplasma infected plants are very apperency within healthy crops.** Plants infected with AYPs have distinctive yellow-green lush foliage and green flowers, rendering them easy to find in a field of crops. Lettuce has yellow and lime green central foliage (A) photograph taken by L. R. Nault ([www.entm.purdue.edu](http://www.entm.purdue.edu)). Echinacea has proliferation of light green foliage and green flowers (B) Photograph from [www.nativecrop.org](http://www.nativecrop.org).

### *1.3.3 Increased oviposition indicates infected plants increase in suitability*

The number of feed/probe sites is significantly lower on AY-WB infected *Arabidopsis* (Fig. 1.2.5.1) than uninfected *Arabidopsis*.

However, the architecture and location of feed/probe sites are unchanged on AY-WB infected and uninfected plants. This may indicate that leafhoppers feed less on AY-WB infected *Arabidopsis* as they may settle more on these plants or may gain more phloem during each probe. The number of feed/probe sites alone provides a limited insight into *M. quadrilineatus* feeding on *Arabidopsis*. A full examination of *M. quadrilineatus* feeding behaviour and any differential feeding on AY-WB infected over uninfected plants could be gained by using Electro penetration graph equipment (Backus *et al.* 2007).

More significant, is *M. quadrilineatus* increased oviposition on AY-WB *Arabidopsis* (Fig. 1.2.4.3 and Fig. 1.2.4.4). Leafhoppers clearly find AY-WB a more suitable host for oviposition than uninfected *Arabidopsis*. Morphological changes in AY-WB infected *Arabidopsis* could explain increase in suitability for oviposition, as *M. quadrilineatus* are more attracted to AY-WB infected *Arabidopsis* (Fig. 1.2.6.1). Increased oviposition could also be explained by changes in nutrient availability in infected plants. Some AYPs alter the carbohydrate concentrations in phloem of sink tissues such as new leaves (Maust *et al.* 2003).

#### *1.3.4 Results in warm temperatures highlights the potential phytoplasma dispersal in crops in ideal conditions.*

*M. quadrilineatus* fecundity is significantly increased on AY-WB infected plants when grown under increased temperature and day length (Fig. 1.2.4.5). This supports previous findings that *M. quadrilineatus* fecundity is affected by temperature as well as phytoplasma infection (Beanland *et al.* 2000; Ebbert and Nault 1999). Larson *et al.*, showed that the black faced leafhopper *Graminella nigrifrons* Forbes develops more rapidly in warmer conditions, but lower temperatures gave rise to larger leafhoppers (Larson *et al.* 1990). Thus, it is likely that warmer temperatures and AY-WB infection may have additive effects on *M. quadrilineatus* on crops.



### 1.3.5 Summary

*Arabidopsis thaliana* provides an elegant model plant to assess phytoplasma manipulations of host plants, due to its short generation time and use in ecological, genomic and proteomic studies. More importantly, in this study it provides an invaluable part of a 3-way model system to study phytoplasmas. In using the fully sequenced AY-WB phytoplasma, efficient vector *M. quadrilineatus* and *Arabidopsis* in this study it is possible to relatively quickly gain insights into how phytoplasmas manipulate their host plants, host interactions and disperse in nature. Using this model system I show here that AY-WB infection in *Arabidopsis*, renders the plants more susceptible to *M. quadrilineatus* oviposition. Feeding is largely unaltered by AY-WB in the plant, suggesting that adult leafhoppers view the plants as more favourable hosts for *M. quadrilineatus* nymphs. Changes in plant morphology suggest that leafhoppers may be attracted to the light green lush new foliage of infected plants rather than healthy plants. Interestingly, it highlights a need for further examination of whether AY-WB altered morphology attracts leafhoppers, or the AY-WB infected plants are easier to find (difference between attraction and apparency).. This work yields clues as to the evolutionary relationship between AY-WB and *M. quadrilineatus*, the effect on *M. quadrilineatus* populations, and the epidemiology of AY-WB in crops.

## **Chapter Two: AY-WB infection within leafhopper vector increases its fecundity**

### **Introduction**

#### *2.1.1 AY-WB infected Arabidopsis are more suitable hosts to M. quadrilineatus*

Aster yellows phytoplasma strain witches' broom (AY-WB) is transmitted to plants hosts including *Arabidopsis thaliana* by the vector leafhopper *M. quadrilineatus*. Although not a reported host plant of *M. quadrilineatus*, the model plant *Arabidopsis* shares geographical distribution to in Northern USA and Canada. The Leafhopper vector *M. quadrilineatus* feeds, reproduces and survives very well on *Arabidopsis* in lab conditions and transmits AYPs to closely related crop host species. I have demonstrated that *M. quadrilineatus* females oviposit more on AY-WB infected *Arabidopsis* over healthy *Arabidopsis* (Fig. 1.2.3.2 [chpt1]). Furthermore, both males and females visit AY-WB infected *Arabidopsis* more than healthy *Arabidopsis* when given the choice. It is unclear whether this choice is due to the preference or greater apparency of AY-WB infected *Arabidopsis*. However, changes induced by AY-WB infection render *Arabidopsis* more susceptible to vector selection and oviposition. These experiments have not alluded to the impact of AY-WB within the insect.

#### *1.1.2 Phytoplasmas have a variety of impacts in their insect hosts*

At least 100 leafhopper, planthopper and psyllid vectors are utilized by phytoplasma to disperse worldwide (Weintraub and Beanland 2006). Insect vector hosts are manipulated to various degrees by phytoplasma infection. The manipulations are thought to be indicative of co-evolutionary relationships of the insect and phytoplasma (Nault 1989; Purcell 1971). For instance, phytoplasma infection in some cases can alter insect fitness. The AYP (group B) maize bushy stunt phytoplasma (MBSP) infection increases longevity of vector *D. maidis* (Nault. 1991).

In addition, MBSP infected females exhibit decreases oviposition, oviposit earlier than healthy females, and increased temperature further reduces oviposition (Madden *et al.* 1984). Two independent strains of Flavescence dorée phytoplasma negatively effect the fecundity of *Euscelidius variegatus* (Bressan *et al.* 2005). Interestingly, plant host change increased the fecundity of this polyphagous leafhopper and counteracts the phytoplasma effect (Bressan *et al.* 2005). Beanland *et al.*, found that AYP strains 'Bolt' and 'Severe' had different effects on the fitness of vector leafhopper *M. quadrilineatus*. AYP 'Bolt' increased survival of *M. quadrilineatus* and AYP 'Severe' increased survival and fecundity of infected leafhoppers (Beanland *et al.* 2000).

Interestingly studies are now emerging of phytoplasmas influencing insect behaviour and plant host choice. Boir noir (BN) vector *Hyalesthes obsoletus* feeds on and vectors BN to nettle and bindweed as reservoir plants surrounding vineyards in Europe. In Switzerland, *H. obsoletus* transmits BN strains tuf-type a to nettle and tuf-type b to bindweed. However, here *H. obsoletus* infected with both BN strains oviposit significantly more on nettle than bindweed (Kehrli *et al.* 2010). In Germany, distinct populations of *H. obsoletus* appear to be feeding and transmitting BN to either Nettle or bindweed in vineyards (Grube *et al.* 2010; Maixner 2010). Grube *et al.* also show that differences are observed between vibrational signal calls of BN infected and uninfected *H. obsoletus* (Grube *et al.* 2010). Phytoplasmas infection within their insect hosts can affect insect fitness, behaviour, interactions with plants and other insects, and speciation between populations. These changes can have impacts on effective phytoplasma dispersal by their vectors.

### 1.1.3 Density of AYP infected leafhoppers within populations fluctuates between populations and years

Known phytoplasmas and vectors are monitored around the world to warn farmers and improve control. Both migratory and sedentary

vector species exhibit fluctuations in population numbers and infective individuals geographically, annually and through the season. These fluctuations increase complexity of prediction of future outbreaks. Local sedentary populations within an area can have different numbers of infected individuals. In northern Italy, *Candidatus phytoplasma mali* infected individuals account for differential proportions over local populations of *Cacopsylla melanoneura* (Malagnini *et al.* 2008). Populations found in 5 areas of the Trentino region were found to have between 3 and 28% infected individuals within their populations. Furthermore, the number of infected individuals was not correlated with the level of symptomatic trees in orchards. Orchards with most symptomatic trees had 11% infected psyllids (Malagnini *et al.* 2008). In earlier years, lower infected numbers and less symptomatic trees were recorded (Mattedi *et al.* 2008).

In the case of migratory vector populations, numbers of infective individuals are influenced by many factors. Geographic dispersal of vectors is weather dependent as many populations travel on storm fronts (Hoy *et al.* 1992). In the great lake region in Canada, AYP infected individuals within aster leafhopper populations vary year on year and do tend to correlate with transmission of phytoplasmas to lettuce (Goodwin *et al.* 1998). In addition, infective individuals can be influenced by temperature. 1995 was exceptional in Canada as numbers of infected leafhoppers were significantly higher than the incidence of AYPs in lettuce (Goodwin *et al.* 1998). The 1995 season temperature was over 35°C for a prolonged period, this significantly reduced successful phytoplasma transmission to plants (Goodwin *et al.* 1998; Kunkel *et al.* 1939; 1941; Murrall *et al.* 1996).

The fitness of the insect also affects the transmission of phytoplasmas. The age of *M. quadrilineatus* affects its transmission efficacy of AYP 'Bolt' and AYP 'Severe' (Murrall *et al.* 1996). Palermo *et al.* found that *M. quadripunctulatus* and *Euscelidus varigatus* lost infectivity for periods, and that AYP acquisition efficiency decreased with age (Palermo *et al.* 2001). The transmission of AYPs also depends on the distribution of

phytoplasma within the insect (Galetto *et al.* 2009; Marzachi and Bosco 2005). The behaviour of the insect populations also affects the dispersal and likelihood of acquisition. Migrant insects movement between crops is dependent on plant species, senescence, insect sex, and time of day (Maixner 2010; Todd *et al.* 1991; Beanland *et al.* 1999; 2005). AYPs also affect population numbers and indirectly dispersal and proportion of infective individuals (Sugio *et al.* 2011; Beanland *et al.* 2000). *D. maidis* females infected with MBSP oviposit more eggs and earlier than healthy females (Madden *et al.* 1984). Phytoplasma manipulations of insect vectors indirectly affect insect population dynamics, thus potentially altering proportions of infective insects within populations through the season.

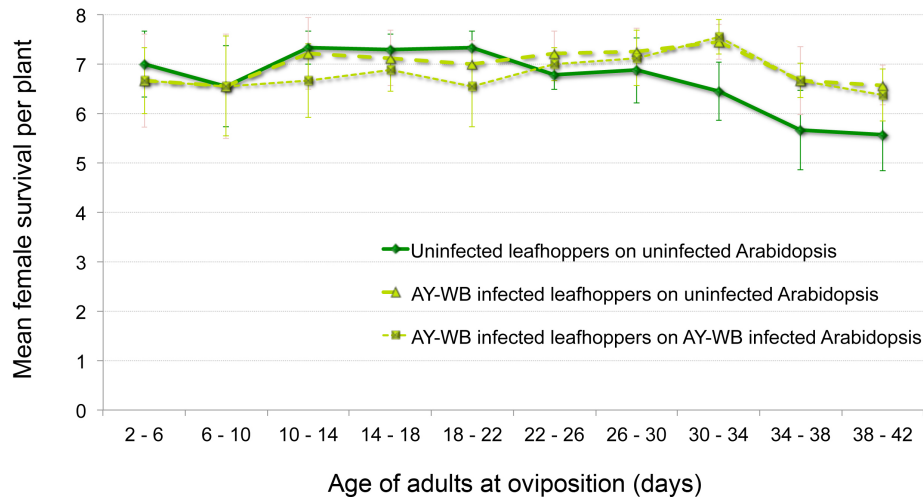
#### *4.1.4 Distinguishing the AY-WB impact on leafhoppers and plants within interactions*

AY-WB is known to have an effect on the fecundity of infected females when infection is in the plant (Fig. 1.2.3.2 [Chpt1]) and when both plant and insect are infected (Beanland *et al.* 2000). However, the impact of AY-WB infection within only the insect has not been properly investigated. Understanding of AY-WB manipulation of host vectors will give an insight as to how phytoplasmas change insect population ecology and the knock on impact on phytoplasma epidemiology. In this chapter, I demonstrate that AY-WB within the insect increases female oviposition on healthy *Arabidopsis*. Furthermore, oviposition is only increased after a latent period of 10 days within the insect. AY-WB has potential to disturb the population ecology of *M. quadrilineatus* by expanding its population growth in future generations. Thus, extending AY-WB dispersal in nature.

## Results

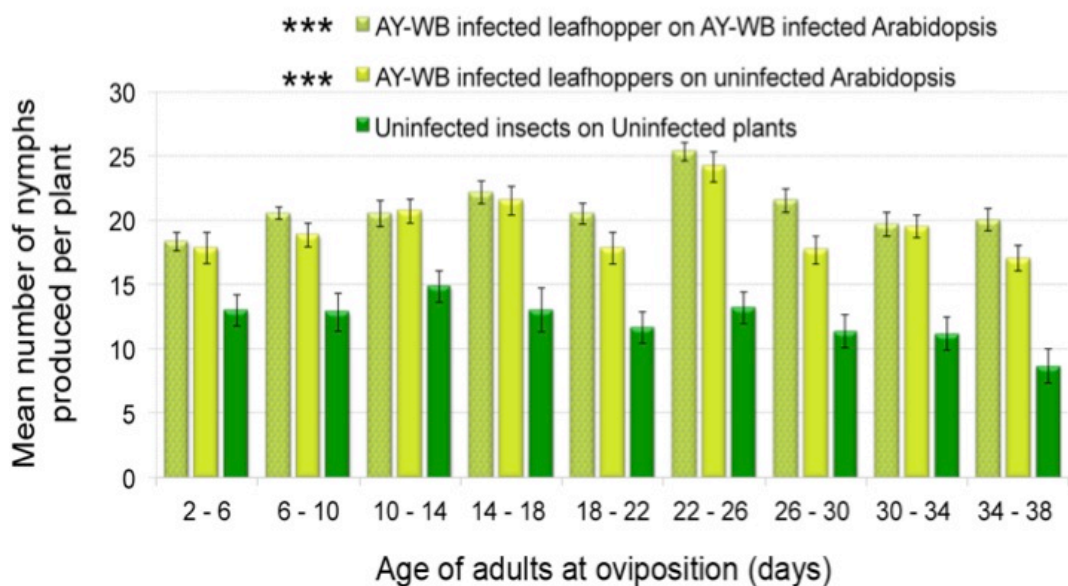
### *2.2.1 AY-WB infection in M. quadrilineatus increases leafhopper fecundity.*

It has been previously reported that aster yellows phytoplasma infection in *M. quadrilineatus* has a positive effect on leafhopper longevity (Beanland *et. al.* 2000). To test if the AY-WB infection in the leafhopper has any effect on *M. quadrilineatus* fitness, survival and fecundity trials were completed using infected leafhoppers. *M. quadrilineatus* were either raised on AY-WB infected or uninfected Arabidopsis and 2 days post eclosure, were added to time series experiments. AY-WB infected and uninfected leafhoppers were added to uninfected Arabidopsis for 4 days after which male and female survivors were counted and removed. Survival was very high on all plants and no significant difference was seen in leafhopper survival (Fig. 2.2.1.1). Nymphs were counted 15 days after adult removal as a measure of fecundity. AY-WB produced significantly more nymphs than uninfected leafhoppers (Fig. 2.2.1.2). AY-WB infected leafhopper oviposition was significantly higher than uninfected leafhoppers (Fig. 2.2.1.3). Hatch rate of eggs oviposited was unaltered (Fig. 2.2.1.4), indicating that increased oviposition was responsible for increase in AY-WB infected *M. quadrilineatus*. Interestingly, oviposition of AY-WB infected insects on AY-WB infected Arabidopsis was not significantly higher than AY-WB infected insects on healthy Arabidopsis (Fig. 2.2.1.2). This indicates that there is no additive effect when both the insect and the plant are infected with AY-WB.

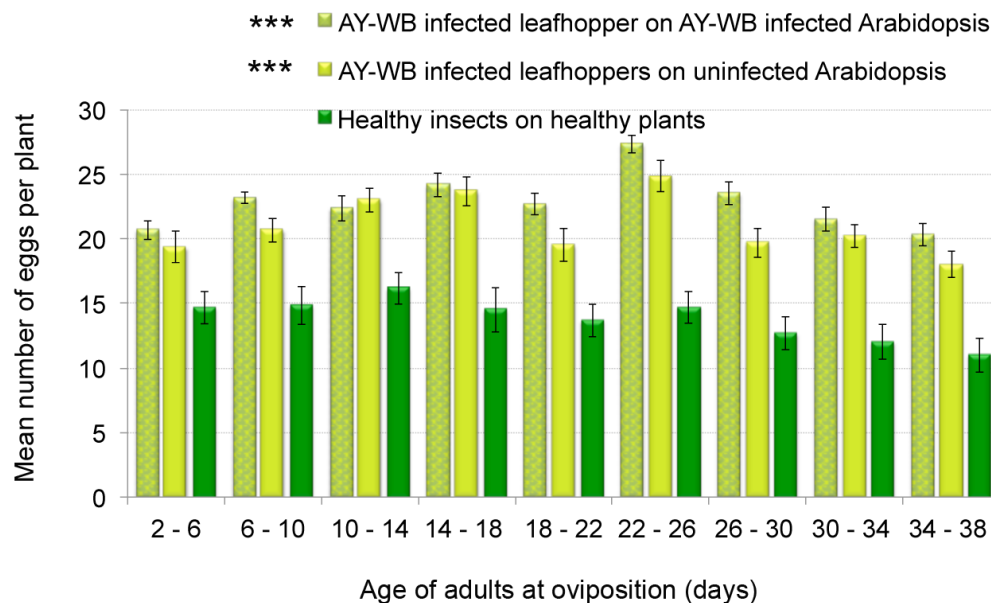


**Figure. 2.2.1.1. *M. quadrilineatus* survival is unaltered by AY-WB infection**

Uninfected leafhoppers were enclosed for 4 days on AY-WB infected Arabidopsis or uninfected inoculated Arabidopsis and male and female survival was recorded. *M. quadrilineatus* has high survival on Arabidopsis over 4 days and as such there was no significant difference in survival of AY-WB infected and uninfected *M. quadrilineatus* on AY-WB infected or uninfected Arabidopsis  $F_{2,109} = 1.733$   $P = 0.177$  ('treatment' in the model). The age of insects on experimental plants had a highly significant effect on *M. quadrilineatus*  $F_{9,109} = 3.01$   $P = 0.001$ . The influence of interactions between female age and treatments on female survival was also insignificant  $F_{1,109} = 0.6$   $P = 0.905$ . The experiment included 10 leafhoppers per plant and 3 plants per treatment per time point. The experiment was repeated 3 times. Bars = 2 x standard error. Model analysed = constant\*replicate\*treatment\*age+Female survival.

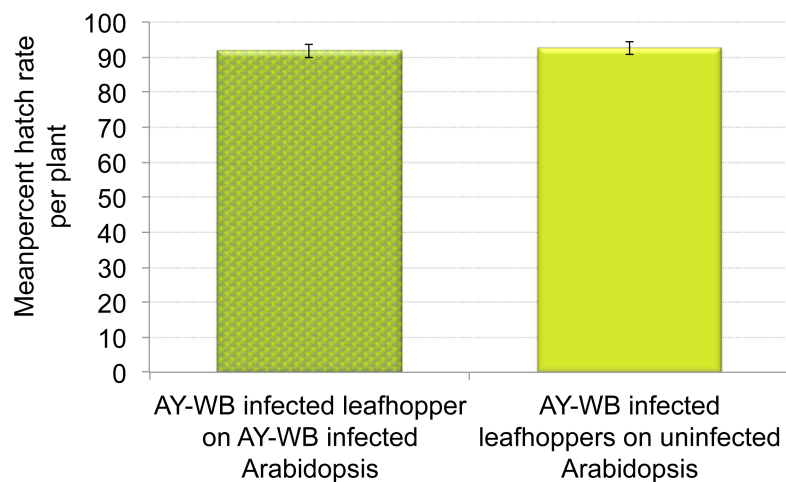


**Figure. 2.2.1.2 *M. quadrilineatus* has increased fecundity on AY-WB infected Arabidopsis.** Leafhoppers produce more nymphs on AY-WB-infected plants. AY-WB-infected and uninfected Arabidopsis were obtained by exposing 5 week-old plants to three AY-WB infected and non-infected male *M. quadrilineatus*, respectively, for one week. Two weeks later 8 adult females and 2 adult males of different age ranges for 4 days (x-axes) were added to experimental plants. Nymphs were counted 15 days after removal of the adult insects. Significantly more nymphs were recorded on plants infected with AY-WB or exposed to AY-WB infected insects  $F_{2,63} = 64.077$   $P = <0.001$  ('treatment' in the model). The age of insects at oviposition also had a highly significant effect on nymph production  $F_{9,63} = 19.76$   $P = <0.001$ . The influence of female survival on nymph production was insignificant  $F_{1,63} = 0.41$   $P = 0.663$ . Each experiment included 10 insects per plant and 3 plants per age range per treatment and the experiments were repeated three times Bars= 2 x standard error, \*\*\*F is highly significant. Model analysed = constant\*replicate\*treatment\*age+Female survival.



**Figure. 2.2.1.3. *M. quadrilineatus* has increased oviposition on AY-WB infected Arabidopsis.** Leafhoppers oviposit more on AY-WB-infected plants. Numbers of eggs were counted one day after removal of the adult insects. Significantly more eggs were found on Arabidopsis infected with AY-WB or exposed to AY-WB infected insects  $F_{2,103} = 59.66$   $P = <0.001$  ('treatment' in the model). The age of insects at oviposition also had a highly significant effect on oviposition  $F_{9,103} = 20.32$   $P = <0.001$ . Each experiment included 3 plants per age range per treatment and the experiments were repeated twice. Bars = 2 x standard error, \*\*\*F is highly significant. Model analysed = constant\*replicate\*treatment\*age.



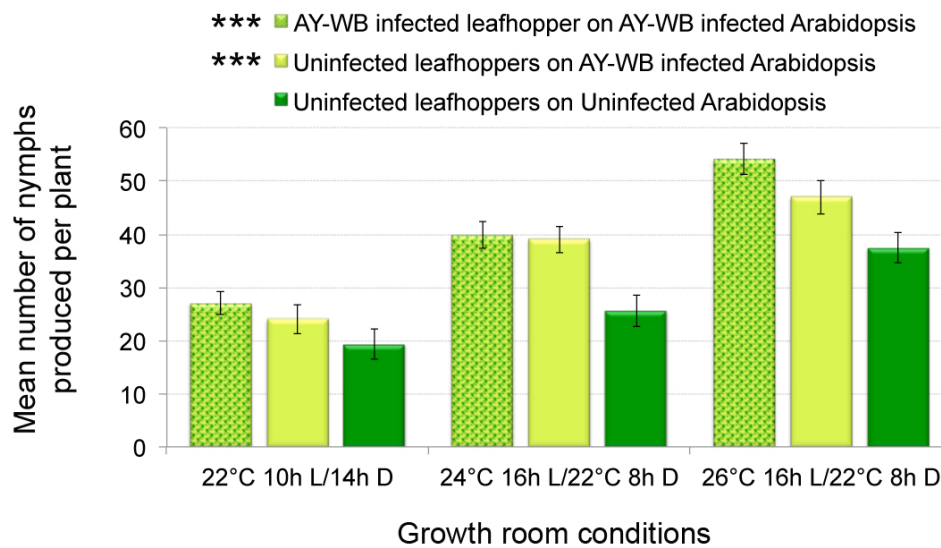


**Figure. 2.2.1.4. AY-WB infected *M. quadrilineatus* egg hatch rate is similar on uninfected and AY-WB infected Arabidopsis.** Leafhopper hatch rate was calculated by dividing the number of eggs by the number of nymphs hatching out after 15 days, giving percentage data. The hatch rate of AY-WB infected *M. quadrilineatus* on AY-WB infected Arabidopsis was not significantly different from that on uninfected Arabidopsis  $F_{1, 68} = 7.46$ ,  $P = 0.703$  ('treatment' in model). The age of insects at oviposition had no significant effect on the hatch rate of *M. quadrilineatus* eggs  $F_{9, 68} = 11.32$ ,  $P = 0.411$ . Experiments included 3 plants per age range per treatment and the experiments were repeated twice. Bars = 2 x standard error. Model analysed = constant\*replicate\*treatment\*age.

## 2.2.2 AY-WB infected *M. quadrilineatus* fecundity is further increased in warmer climate conditions

Field conditions in which leafhoppers such as *M. quadrilineatus* thrive, are warmer than the experimental conditions outlined here. In order to gain an indication of how fecundity may be affected on AY-WB infected Arabidopsis in warm conditions, like those encountered by *M. quadrilineatus* during migrations, fecundity experiments were repeated in three different conditions. Controlled growth rooms were used to ensure constant conditions. room A was set, as previously, to cool conditions at 22°C consistently with a 10 hour day and 14 hour night. Room B was warmer at 24°C 16 hour day and 22°C 8 hour night and room C was warmer still with 26°C 16 hour day and 22°C 8 hour night. 14-18 day-old leafhoppers were used on experimental AY-WB infected

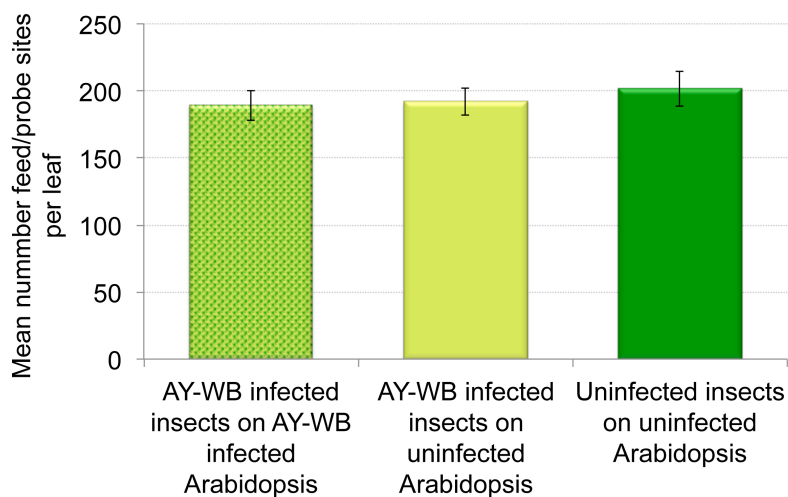
or uninfected *Arabidopsis* plants. *M. quadrilineatus* had high survival in all conditions with no difference between conditions. Fecundity was increased on AY-WB infected *Arabidopsis* in all conditions. Furthermore, fecundity increased significantly in each room with increase in temperature and day length on both uninfected and AY-WB infected *Arabidopsis* (Fig. 2.2.2.1). Variability in fecundity did increase with increasing temperature, however, this difference was not significant. These data indicate that warmer conditions have a significant increasing effect on fecundity.



**Figure. 2.2.2.1. *M. quadrilineatus* fecundity is increased in warmer climate conditions.** Fecundity was re-examined in 14-18 day old leafhoppers, either infected with AY-WB or uninfected on uninfected or AY-WB infected *Arabidopsis*, in three different environmental conditions. Conditions were 22°C with a 10 hour day and 14 hour night, 24°C 16 hour day and 22°C 8 hour night, and 26°C 16 hour day and 22°C 8 hour night. Fecundity was significantly increased on AY-WB infected plants and when *M. quadrilineatus* was infected with AY-WB,  $F_{2,36} = 23.275$   $P < 0.001$ . *M. quadrilineatus* in all treatments had significantly increased nymph production in rooms with higher temperatures,  $F_{2,36} = 68.274$   $P < 0.001$ . The experiment included 10 leafhoppers per plant on 12 plants per treatment, per room condition ('temperature' in model) and the whole experiment was repeated twice. Bars = 2 x standard error. Model analysed = constant\*replicate\*treatment\*temperature.

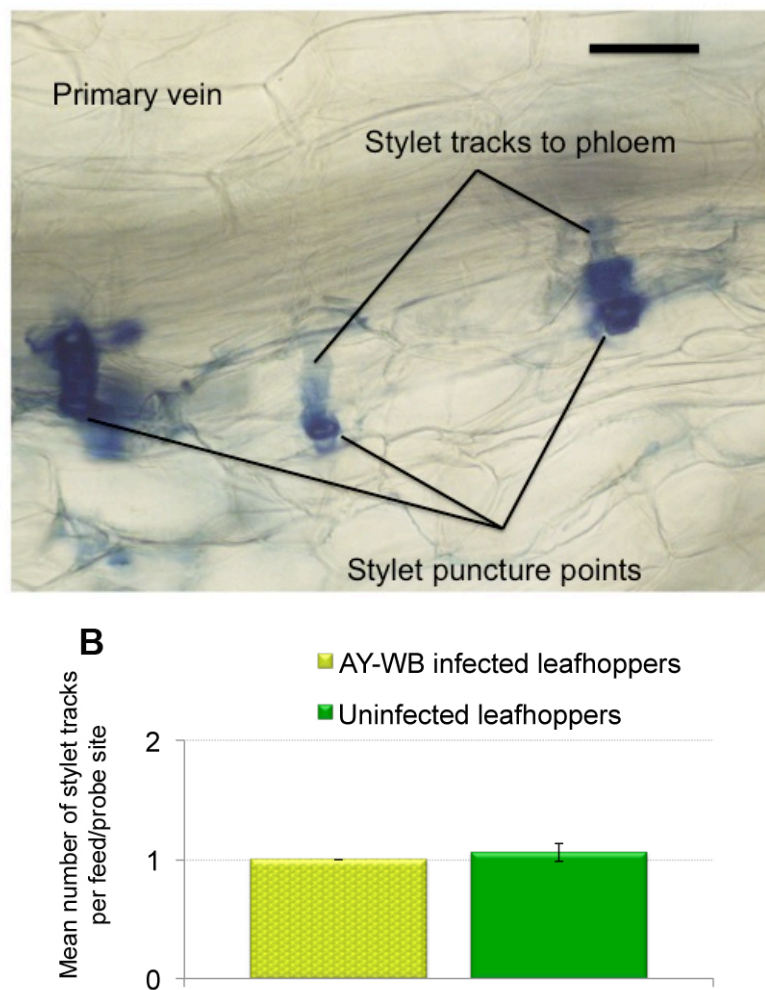
### 2.2.3 AY-WB Infection in leafhoppers does not affect feeding on *Arabidopsis*

*M. quadrilineatus* is able to feed effectively on *Arabidopsis*, feeding mainly from central veins and using single stylet tracks towards phloem. To examine any impact AY-WB infection has on the feeding of *M. quadrilineatus*, AY-WB infected leafhoppers and uninfected leafhoppers were enclosed on single uninfected *Arabidopsis* leaves for 5 days. Leaves were then cut and stained with trypan blue to highlight feed/probe sites and examined leaves under a Nikon E800 fluorescent microscope. AY-WB infected leafhoppers showed no difference in feed/probe site levels on uninfected or AY-WB infected *Arabidopsis* leaves (Fig. 2.2.3.1). In addition, AY-WB infected *M. quadrilineatus* showed no difference on feed/probe site morphology for uninfected leafhoppers (Fig. 2.2.3.2). These data indicate that AY-WB infection within the leafhopper has no effect on the feeding ability of these leafhoppers.



**Figure. 2.2.3.1. AY-WB infected *M. quadrilineatus* exhibit uniform feeding on *Arabidopsis* regardless of plant infection.** AY-WB infected and uninfected *M. quadrilineatus* were enclosed on AY-WB infected and uninfected single leaves ('treatment' in the model) for 5 days and removed. Leaves were cut and stained with trypan to render feed/probe sites marked in blue. Total feed/probe sites were counted per leaf and their location recorded. No significant difference was found in numbers of feed/probe sites per leaf in any treatment  $F_{2,24} = 1.879$ ,  $P = 0.313$ . The experiment included examination of 12 leaves per treatment exposed to 5 leafhoppers

each. The whole experiment was repeated twice. Bars = 2 x standard error. Model analysed = constant\*replicate\*treatment.

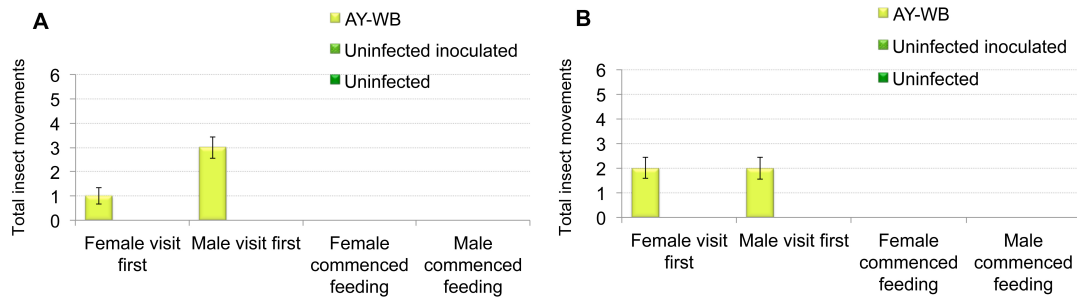


**Figure. 2.2.3.2. AY-WB infected *M. quadrilineatus* feeding damage is identical uninfected *M. quadrilineatus*.** AY-WB infected and uninfected *M. quadrilineatus* were enclosed on AY-WB infected and uninfected single leaves for 5 days and removed. Leaves were cut and stained with trypan to render feed/probe sites highlighted in blue. Feed/probe sites were observed under a Nikon E800 Fluorescent microscope and puncture points and stylet tracks per feeding sites were recorded in 10 circular quadrats per leaf. **A** The majority of feed/probe sites consisted of only one puncture point and one feeding track towards phloem. **B** No significant difference in numbers of stylet tracks per puncture points was observed in feed/probe sites on AY-WB infected and uninfected leafhoppers  $F_{1, 27} = 3.1181$ ,  $P = 0.720$ . Bar = 10 microns. The whole experiment included 6 leaves per treatment and 10 quadrats per leaf were examined.

The experiment was repeated once. Bars = 2 x standard error. Model analysed = constant\*replicate\*treatment.

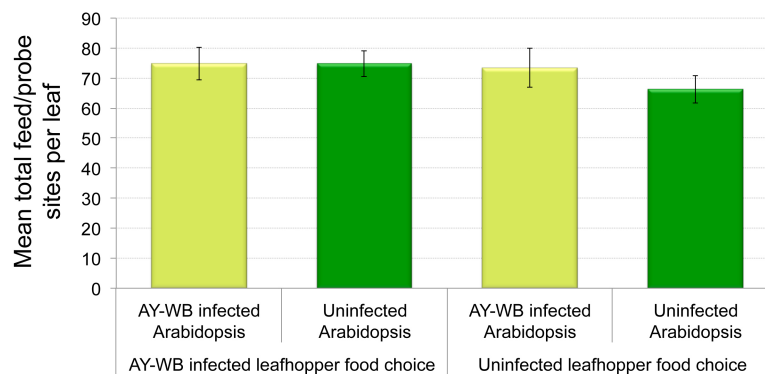
#### 2.2.4 *Infected leafhoppers showed no host preference*

In order to ascertain whether AY-WB infection within the leafhopper could affect host choice, choice chamber experiments were repeated with AY-WB infected *M. quadrilineatus*. Leafhoppers were individually placed in a choice chamber for 15 minutes and observed. Within the chamber were; an AY-WB infected Arabidopsis, an untreated Arabidopsis, and an uninfected inoculated Arabidopsis. The choice tests were completed in white light to test whether choice was affected by visual cues. The 1st plant visit and the plant first settled on to feed/probe were recorded. In most repeats leafhoppers groomed for the full 15 minutes of the test and did not move toward any plant. Of those that did move, all visited AY-WB infected plants. However, there was no significant preference found on analysis of the data (Fig. 2.2.4.1). This suggests the observational time was not sufficient in this experiment. To re-examine this, a 2day experiment was carried out and included uninfected *M. quadrilineatus*. Five AY-WB infected leafhoppers were enclosed in 15ml falcon tube cages with access to 2 AY-WB infected Arabidopsis leaves at one end and 2 healthy Arabidopsis leaves at the other end. Leaves were cut from plants after 2 days exposure, stained with trypan blue and mounted in 80% glycerol on slides for observation. Leaves were examined under a Nikon E800 fluorescence microscope and feeding sites and eggs were recorded on each leaf. No significant feeding increase was observed on AY-WB or healthy leaves (Fig. 2.2.4.2). In addition no eggs were found on any exposed leaves. These data highlight that AY-WB infection within the insect has no effect on host preference, but AY-WB within the plant does make Arabidopsis more attractive or more suitable to uninfected *M. quadrilineatus* (Fig. 2.2.4.2).



**Figure. 2.2.4.1. AY-WB infected *M. quadrilineatus* do not behave as expected in choice chambers**

A choice chamber experiment was used to determine host plant preference of AY-WB infected leafhoppers. Plants were replaced in the chamber after alternate tests. Whether a female or male entered the chamber first was recorded as a potential variable. Most female and male leafhoppers did not move from where they were placed in the choice chamber in the allocated time of 15 minutes per test. All insects groomed for the majority of the experiments. No significant preference was recorded  $P=0.418$ . A total of 6 females and 6 males were placed in the choice chamber with a new set of plants on alternate tests. The whole experiment was repeated twice. Data were analysed using Chi-square analysis.

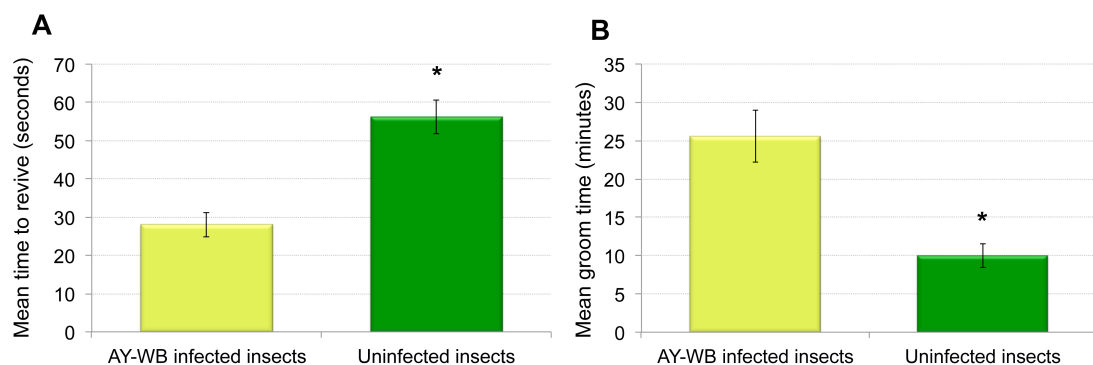


**Figure. 2.2.4.2. Uninfected *M. quadrilineatus* show preference for AY-WB infected Arabidopsis.** Insects were exposed both to uninfected and AY-WB infected leaves and allowed to feed from either for 2 days. Leaves were then cut, stained with trypan blue, and feed/probe sites were observed under a Nikon E800 fluorescent microscope. AY-WB infected *M. quadrilineatus* showed no significant feeding preference when given access to uninfected or AY-WB infected Arabidopsis  $F_{1,24} = 8.723$   $P = 0.304$ . Uninfected individuals fed significantly more on AY-WB infected Arabidopsis leaves than on uninfected leaves,  $F_{1,24} = 22.4207$   $P = 0.01$ . Experiment

included 3 replicates per treatment and the whole experiment was repeated 4 times. Bars = 2 x standard error. Model analysed = constant\*replicate\*treatment.

### 2.2.5 AY-WB infected *M. quadrilineatus* exhibit altered grooming behaviour

Whilst conducting other experiments I noticed differences in behaviour between AY-WB infected and uninfected leafhoppers. When knocking out leafhoppers with CO<sub>2</sub> to sex them, AY-WB infected leafhoppers appeared to revive more quickly than uninfected individuals. *M. quadrilineatus* also groomed themselves much longer than uninfected leafhoppers when moved to a new cage. To try and quantify these differences in behaviour, I collected mixed sex groups of 20 AY-WB infected or uninfected leafhoppers in flasks and conducted two experiments. Leafhoppers inside flasks were anesthetized with CO<sub>2</sub> treatment, and their revival was timed. In a second experiment fresh leafhoppers were moved, in mixed sex groups of 20, to new cages and their initial behaviour was recorded. AY-WB infected *M. quadrilineatus* were awake in 30 seconds; half the time taken by uninfected leafhoppers to wake (Fig. 2.2.5.1A). In addition, after leafhopper relocation to new cages, the duration of grooming by AY-WB infected insects was more than double that of uninfected leafhoppers (Fig. 2.2.5.1B). These data highlight that AY-WB infection within the insect can have an effect on grooming behaviour and the recovery from CO<sub>2</sub> Anesthesia.



**Figure. 2.2.5.1. AY-WB infected *M. quadrilineatus* have altered behaviour**

Mixed sex groups of 20 AY-WB infected and uninfected leafhoppers were placed in flasks, knocked out with CO<sub>2</sub>, and allowed to revive. The difference in revival time of *M. quadrilineatus* in these conditions was highly significant  $P = <0.01$ . Leafhoppers infected AY-WB revived in approximately 30 seconds whereas uninfected leafhoppers took nearly 60 seconds to revive (A). Mixed sex groups of 20 were moved from one cage to another and their grooming behaviour was recorded. Grooming behaviour of AY-WB infected leafhoppers was also highly significantly different to that of uninfected leafhoppers  $P = <0.01$ . AY-WB infected insects spend more than 25 minutes grooming when moved into a new cage, whereas uninfected *M. quadrilineatus* groomed for only 10 minutes (B). Experiments A and B included 20 insects per enclosure and both experiments were repeated 30 times. Data was analysed using student t tests. Error bars shown are 2x standard error above and below means.

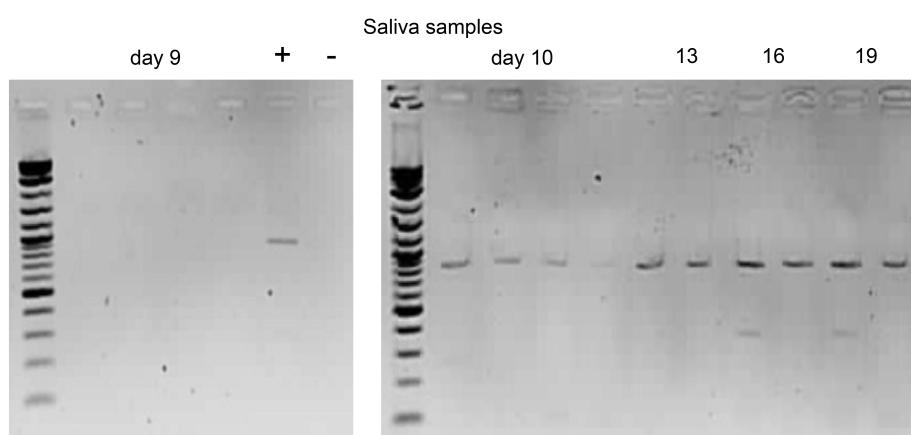
#### 2.2.6 AY-WB infection reaches *M. quadrilineatus* salivary glands, saliva, and ovaries 10 days after infection

AY-WB has an effect on fecundity 10 days after phytoplasma acquisition. It is likely that the phytoplasma location within the insect and/or the titre must be relevant to any effect on the insect host. Furthermore, AY-WB is found in the saliva 10 days after acquisition. Kelly Bennett, an MSc student under my supervision conducted a PCR assay of infected *M. quadrilineatus*. She found that AY-WB is present only after 9 days incubation in the insect (Fig. 2.2.6.1). This indicates that at the infection stage fecundity is increased (after 10 days), AY-WB is present in saliva and potentially transmitted to the plants. Therefore, AY-WB in the insect only increases fecundity at the point *M. quadrilineatus* can vector AY-WB into the plant. This suggests that as AY-WB is transmitted to plants, almost immediately it could cause increased fecundity of *M. quadrilineatus*.

To assess the dispersal of AY-WB throughout the leafhoppers after 10 days of infection leafhoppers were dissected at this point to obtain reproductive organs, salivary glands and haemolymph. Tissues from 20 leafhoppers were mixed to generate enough material to assay with PCR using AY-WB specific primers (Zhang *et al.* 2004). AY-WB was present

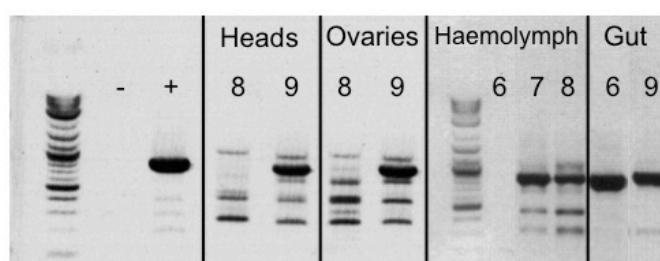


in all tissues in line with findings of Ammar *et al.* 2003 (Fig. 2.2.6.2). Once AY-WB moves from the gut to the haemolymph it moves to the reproductive organs, salivary glands and potentially other organs. This dispersal around the insect highlights the potential to affect leafhopper fecundity.



**Figure. 2.2.6.1. AY-WB reaches the salivary glands and is secreted in saliva after 9 days of incubation in *M. quadrilineatus*.**

AY-WB presence in saliva of *M. quadrilineatus* was assessed by PCR after exposing leafhoppers to AY-WB infected Arabidopsis over a time series of 20 day. Saliva was collected using feeding membrane cages with 5% sucrose between membranes. Saliva in sucrose solution was subjected to colony PCR assays with AY-WB specific primers. Saliva from leafhoppers 9 days after exposure to AY-WB infected plants, produced amplicons of the expected 900 base pair size. AY-WB phytoplasma was found in saliva from all tested saliva 9 days after exposure to AY-WB. Saliva samples pooled from 5 cages per time point and experiment repeated twice. Work completed by Kelly Bennett (MSc student under my supervision).



**Figure. 2.2.6.2. AY-WB is found in insect organs 9 days after acquisition.**

Post acquisition, *M. quadrilineatus* organs were dissected, pooled and assessed by PCR using universal phytoplasma primers P1/P7 for presence of AY-WB. Leafhoppers were exposed to AY-WB infected Arabidopsis over a time series of 10 days. The

expected 900 base pair bands were seen in all gut tissue every day post acquisition (p/a). AY-WB was found in haemolymph 7 days p/a, ovaries 9 days p/a, and salivary glands 9 days p/a. 10 insects (20 for haemolymph) were used in sample mixes used in PCR assays for each day. The experiment was repeated twice.

## Discussion

### *3.3.1 Increased oviposition of infected females indicates manipulation of vector insect.*

*M. quadrilineatus* show increased oviposition on AY-WB infected Arabidopsis (Fig. 1.2.4.2). Interestingly here, AY-WB infected vector females have increased nymph production on uninfected Arabidopsis (Fig. 2.2.1.2). AY-WB infected leafhoppers in these experiments had been reared on AY-WB infected Arabidopsis, thus AY-WB had had time to accumulate throughout the insect body. It is possible that this accumulation alters insect oviposition or egg production from within the insects. AY-WB must have at least 10 days to accumulate within the insect to change leafhopper nymph production, as shown in chapter one (Fig. 1.2.3.1). However, at this point AY-WB phytoplasmas can be found in infected leafhopper saliva (Fig. 2.2.6.1). Indicating that either AY-WB is manipulating the plant immediately after initial inoculation, or that the female insect egg production or oviposition is manipulated from within by AY-WB infection.

In other investigations of AY-WB infection in Arabidopsis plants, one week after initial infection, AY-WB is found throughout plant sink tissues (Ammar *et al* 2006; Hogenhout *et al.* 2008). The candidate effector protein SAP11 responsible for manipulation of plant defence (discussed in chapter 4) has been found throughout Arabidopsis seedling roots after one-week infection (unpublished data from Hogenhout lab, not shown). In microarray data, AY-WB infection in Arabidopsis showed minimal transcriptional changes in the plant,

whereas, after 2 weeks infection transcriptional changes were highly significant (unpublished data from Hogenhout lab, not shown). It is therefore as yet not clear that AY-WB is affecting plant hosts immediately after initial inoculation or one or two or more days post infection. To investigate any early AY-WB manipulation of plant hosts, fecundity of *M. quadrilineatus* on Arabidopsis plants in an infection time series would need to be carried out.

Within the insect, AY-WB is found in the salivary glands, saliva, gut, haemolymph and ovaries 10 days post infection (Fig. 2.2.6.1 and 2.2.6.2). *M. quadrilineatus* fecundity can be increased in the insect after 10 days infection (Fig. 1.2.3.1). At which point it is possible that AY-WB accumulation and secretion of candidate effector proteins can modulate egg production, egg maturation or oviposition behaviour. It is plausible that AY-WB phytoplasma secreted proteins interact with insect proteins such as yolk proteins and in turn may affect fecundity. Yolk proteins, regulated by insect hormones, are essential in development of oocytes in insect ovaries and are formed in fat bodies of insects (Izumi *et al.* 1994). Fat bodies are found in insect haemolymph, which is colonized by day 7 in AY-WB infection in *M. quadrilineatus* (Fig. 2.2.6.2). These data present strong evidence that AY-WB infection within the insect has a direct effect on *M. quadrilineatus* fecundity via increased oviposition. Further assessment of AY-WB impact at initial and early infection within that plant needs to be completed to isolate contribution of effect of infection within the plant. Further study of interactions of AY-WB secreted proteins and insect proteins may yield clues as to how this modulation occurs. This potential direct effect on AY-WB infected *M. quadrilineatus* potentially increases next generation of vector insects, thus having an indirect benefit to the dispersal of AY-WB.

### *3.3.2 Lack of host preference indicates infected leafhoppers transmit AY-WB at random to various hosts in nature*

AY-WB infected plants are visited more by healthy *M. quadrilineatus* (Fig. 1.2.6.1). In contrast, AY-WB infected leafhoppers show no such

preference (Fig. 2.2.4.1). AY-WB infected insects plant choice seems random and not dictated by plant attraction or apparency. This indicates that AY-WB infection within the insect alters the insect host location or host choice behaviour. The choice test experiments were not conducted in a Y chamber but a large open square cage with plants all open to the insect. In all choice tests, AY-WB infected leafhoppers groomed for some time before moving towards any plant. In light of extended grooming behaviour of the AY-WB infected insects, I am not confident that the choice experiments give a clear indication of infected insect preference. It would be appropriate to repeat these experiments allowing more time for leafhoppers to settle and move towards a plant. If the data represents a true lack of host preference in infected *M. quadrilineatus*, it is possible that it may affect the spread of phytoplasmas in field conditions. Phytoplasma infection in insects has been shown to affect insect-plant associations (Grube *et al.* 2010, Maixner, 2010, Kehrli *et al.* 2010).

Uninfected *M. quadrilineatus* are known to show preference to crops surrounded by weed grass species rather than broadleaf weeds (Szendrei, 2012). Further experiments on known crop and surrounding weed host plants are needed to examine host location, selection and settling for feeding and oviposition. These data will yield vital clues as to any change in plant associations and behaviour due to phytoplasma infection in the insect vectors. Examining insect choice before and after infection to determine whether host choice is changed by infection and at what point in AY-WB infection this change occurs, could yield clues as to how AY-WB infection affects individual and population host choice.

### *3.3.3 AY-WB manipulation of leafhopper behaviour could contribute to annual fluctuation in numbers of AYP infective individuals within populations.*

*M. quadrilineatus* grooming behaviour and recover from CO<sub>2</sub> is altered by AY-WB infection (Fig. 2.2.5.1). Larson *et al.* and Beanland *et al.*

reported that AYP infected males were more mobile than healthy male *M. quadrilineatus* (Larson *et al.* 1990; Beanland *et al.* 2005). During experiments, I observed that AY-WB infected leafhoppers appeared more active in cages than healthy insects (not quantified). Leafhopper grooming may be a result of many stimuli, change in surrounding, being disturbed, shock, or increasing contact with substrate (Rakitov 1996). AY-WB infected leafhoppers may have a heightened response to shock and new surroundings or a heightened immune response due to AY-WB infection. However, altered grooming behaviour is not known to be relevant to AY-WB transmission. Further quantification of changes in mobility and recovery of leafhoppers will prove important in understanding the contribution of behaviour in AYP transmission to crops. If AY-WB infected leafhoppers are more mobile and fitter than healthy insects, AY-WB individuals may be more successful in a population and contribute to an increase in numbers of infective individuals. Any contribution of altered behaviour by AY-WB infection is likely to be more pronounced in multivoltine species due to population growth with each generation within a season.

### 3.3.3 Summary.

AY-WB infected insects have increased fecundity on uninfected *Arabidopsis*, but only when the phytoplasmas have reached the haemolymph, salivary glands, ovaries and other tissues. This indicates that a certain phytoplasma titre is required to have an effect on fecundity. This raises questions as to the processes that AY-WB phytoplasma and their secreted proteins may be influencing within the insect host. AY-WB may affect the fat bodies, insect hormones, insect protein production, oocyte development, egg maturation or oviposition behaviour, in ways that increase oviposition or egg production or viability of eggs. This needs to be further investigated by studying interactions between AY-WB secreted proteins and *M. quadrilineatus* proteins and localization of AY-WB and AY-WB secreted proteins within *M. quadrilineatus*.

AY-WB infected insects also show indication of modulated behaviour on *Arabidopsis*, such as a significant change plant host selection. Further examination of infected *M. quadrilineatus* host plant selection on known crop and weed plants will yield clues as to the relevance and impact of phytoplasma manipulations of behaviour of its leafhopper vector, and indirect affects on phytoplasma epidemiology.

**Chapter three: The potential of AY-WB and *Dalbulus maidis* as a  
model system in the study of phytoplasma in non-host  
interactions.**

**Introduction**

*3.1.1 Phytoplasma infection influences insect-plant interactions*

The impact of phytoplasmas on interactions between insect vectors and plant hosts are varied. In the case of *Dalbulus* leafhoppers, maize bushy stunt phytoplasma (MBSP) and corn stunt spiropasma (CSS) *Spiroplasma kunkelii* may have influenced the evolution and distribution of its vector leafhoppers via differences in pathogenicity. *D. maidis* has improved over wintering and longevity is increased when infected with either CSS or MBSP. In contrast *D. elimatus* and *D. gelbus* adult life spans are not altered by MBSP in Mexico but can be shortened in other areas and *D. longulus*, *D. guzmani*, *D. tripsacoides*, *D. quinquenotatus*, and *D. guevarai* and all have reduced longevity when infected with CSS or MBSP. The later 3 *sp.* are found only on *Tripsacum sp.*, which are immune to CSS and MBSP, but all *Dalbulus sp.* can feed on maize and vector CSS and MBSP (Nault *et al.* 1989, Nault 1990). Human activity has moved many species into contact with novel hosts, as a result, these interactions can be equally dramatic. *Scaphiodius titanus* is an American leafhopper introduced to Europe around world war II, and the vector of Flavescence Dorée, an elm yellows phytoplasma causing a grapevine yellows. This phytoplasma also has a negative effect on the longevity of the vector leafhopper (Bertin *et al.* 2007). In other cases phytoplasma infection in the plant can alter interactions. Apple proliferation phytoplasma (*candidatus phytoplasma mali*) infected apple trees are manipulated to give off modified volatiles, that attract vector psyllid, *Cacopsylla piri* (Mayer *et al.* 2007). Aster yellows phytoplasma strain witches' broom (AY-WB) manipulates plant host defences causing an increase in longevity and fecundity of its vector leafhopper *Macrosteles quadrilineatus* (Sugio *et al.* in press). Insect-

plant interactions can be affected by phytoplasmas with great variety in results.

### *3.1.2 Phytoplasmas manipulate non-host interactions*

The maize specialist leafhopper *D. maidis* can survive on *Tripsacum* grasses when maize is unavailable, however reproduction is very low (Pitre. 1970). Interestingly, *D. maidis* can survive on aster yellows phytoplasma (AYP) infected aster and lettuce plants but dies on healthy lettuce or aster (Barnes. 1954; Weintraub & Beanland. 2006). *D. maidis* can also be conditioned to have higher survival on healthy aster and lettuce if initially raised on AYP infected lettuce and aster (Purcell. 1987; Weintraub & Beanland. 2006). It is unclear how the phytoplasma is affecting the interaction as *D. maidis* is not a vector of that particular AYP and was unable to transmit it. Understanding how phytoplasmas manipulate non-host interactions would benefit research into adaption to novel insect and plant hosts.

### *3.1.3 Appraisal of D. maidis and Arabidopsis as a model non-host interaction to study phytoplasma manipulation.*

Phytoplasma impact on non-host interactions has never been examined within a model system such as *Arabidopsis thaliana*. I aim to assess whether the AYP aster yellows phytoplasma strain witches' broom (AY-WB) can affect the non-host interaction between *D. maidis* and *Arabidopsis thaliana*. This could provide a valuable tool in the future research of phytoplasma manipulation of hosts, novel insect and plant hosts and potential novel vectors. This is especially relevant in our current time of climate change, human activity and global food security.

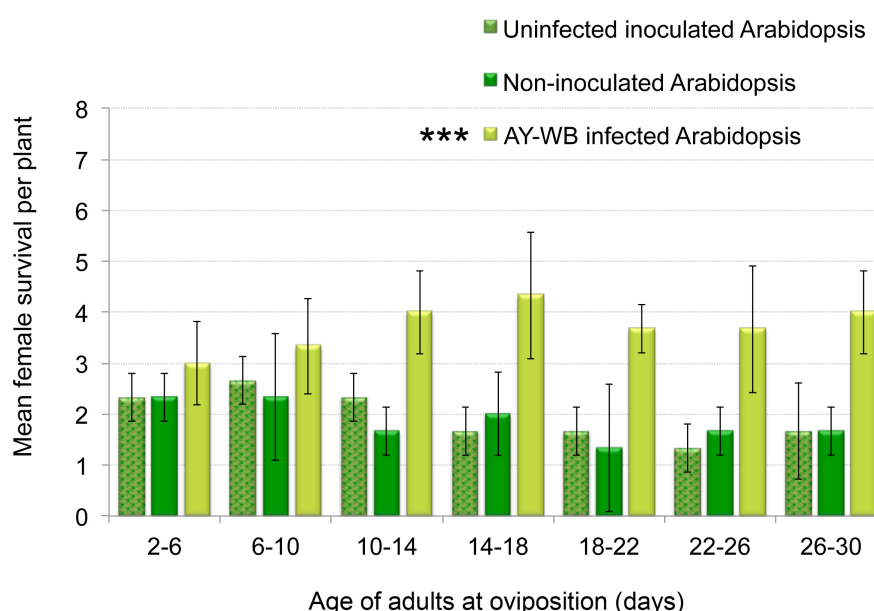


## Results

### *3.2.1 Dalbulus maidis survives and reproduces on AY-WB infected Arabidopsis.*

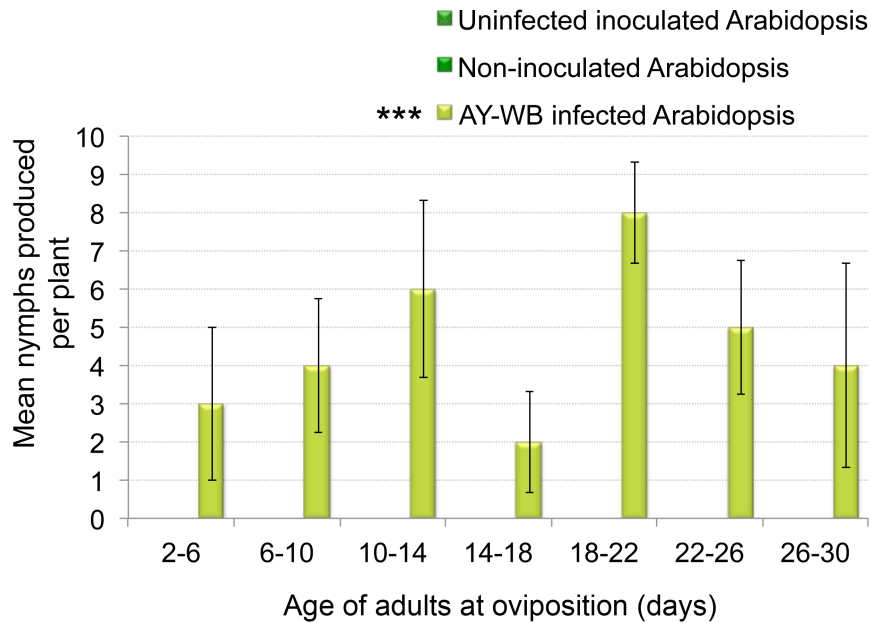
Survival of *D. maidis* was tested on individually enclosed Arabidopsis plants in 3 different conditions. Experimental plants were untreated, inoculated with male AY-WB infected *Macrosteles quadrilineatus* or inoculated with healthy male *M. quadrilineatus*. *D. maidis* consistently showed low survival on untreated and healthy inoculated Arabidopsis but *D. maidis* survival was significantly increased on AY-WB infected Arabidopsis (Fig. 3.2.1.1). Survival was recorded after 4 days of enclosure on plants and live adults were removed. To assess fecundity we retained plants and observed for a further 15 days when any emerging nymphs were counted. No nymphs emerged on untreated or healthy inoculated Arabidopsis and no eggs were found on these plants. Eggs and nymphs were found on many AY-WB infected Arabidopsis plants, which highlights *D. maidis* fecundity is significantly increased on AY-WB infected non-host Arabidopsis (Fig. 3.2.1.2). Survival and fecundity experiments were repeated on 14 day-old *D. maidis* in different conditions to assess the AY-WB impact in warmer climates. Three controlled growth rooms were used with different conditions. Room A was set to 22°C consistently with a 10 hour day and 14 hour night. Room B was set for 24°C 16 hour day and 22°C 8 hour night and room C was 26°C 16 hour day and 22°C 8 hour night. *D. maidis* survival was significantly increased on AY-WB infected Arabidopsis in rooms A and B but not in room C conditions (Fig. 3.2.1.3). In room C conditions insects had increased survival on untreated and uninfected Arabidopsis than in cooler conditions (Fig. 3.2.1.3). AY-WB infection in Arabidopsis and warmer temperatures and longer days increase *D. maidis* survival (Fig. 3.2.1.3). Nymphs and eggs were only on AY-WB infect Arabidopsis and number of eggs and nymphs were not significantly affected by climate conditions (Fig. 3.2.1.4).

Eggs were predominantly located on the underside of mature Arabidopsis leaves along the mid vein. Females oviposited under the epidermal cells leaving a little or no protruding egg through the plant cells (Fig. 3.2.1.5). Eggs remained transparent for the first 1-2 days before turning pale cream and more visible through the epidermal cells. Eggs developed the characteristic wax microfilaments described by Heady and Nault after a few days, which aided location of eggs with a naked eye (Heady and Nault, 1984). *D. maidis* egg hatch rate on AY-WB infected Arabidopsis was 35% (Fig. 3.2.1.6), which is a little higher than the 25% hatch rate of *D. maidis* on *Tripsacum* grasses recorded by Pitre (Pitre., 1970).



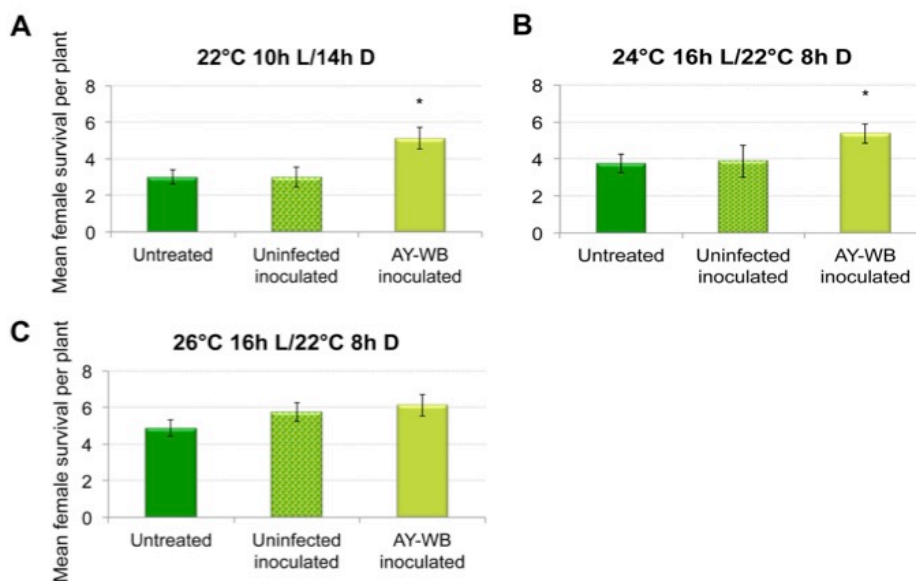
**Figure 3.2.1.1. *D. maidis* female's survival increase on AY-WB infected Arabidopsis.**

*D. maidis* survival was measured on untreated Arabidopsis, Arabidopsis exposed to healthy male *Macrostes quadrilineatus* or Arabidopsis exposed to AY-WB infected male *M. quadrilineatus*. Survival was quantified as the number of surviving females out of 8 after 4 day confinement to each plant. Survival was significantly increased on AY-WB infected Arabidopsis  $F_{2,42} = 13.054$   $P = <0.001$  \*\*\*. Age of the insects had no effect on the survival of these insects  $F_{6,42} = 0.2566$   $P = 0.957$ . Bars show 2X standard error. Modeled by rep\*treatment\*age using GLM with a binomial link function. Error bars shown are 2x standard error above and below means.



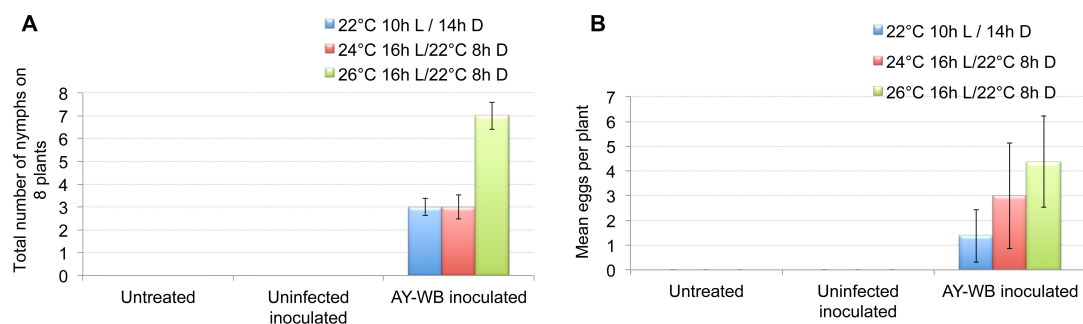
**Figure 3.2.1.2 *D. maidis* fecundity is increased on AY-WB infected Arabidopsis.**

Fecundity was measured as the number of nymphs produced per plant 15 days post adult removal. Nymphs were only present on AY-WB infected Arabidopsis, thus AY-WB infected plants had significantly more nymphs  $F_{2,41} = 35.1546$   $P = <0.001$  \*\*\* and age of the insects at oviposition had no effect on nymph numbers  $F_{6,41} = 0.8571$   $P = 0.526$ . Bars show 2X standard error. Modeled by  $\text{rep} \times \text{treatment} \times \text{age} + \text{female survival}$  using GLM with a poisson link function. Error bars shown are 2x standard error above and below means.



**Figure 3.2.1.3. *D. maidis* female survival is further increased on AY-WB infected Arabidopsis in warmer conditions.**

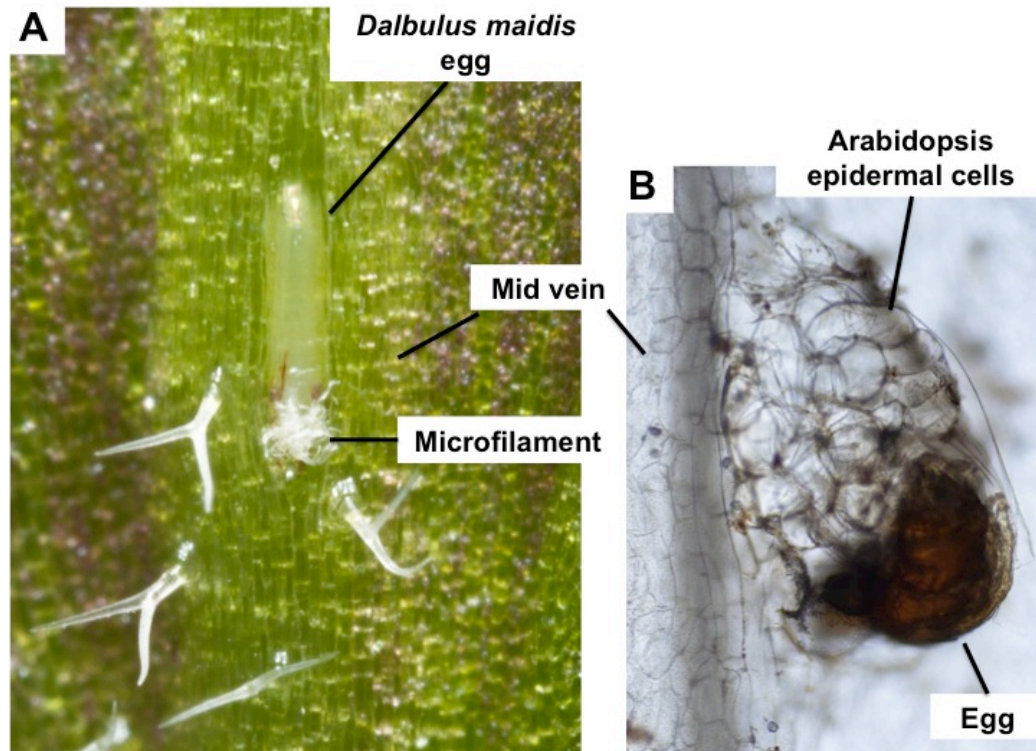
*D. maidis* survival was measured on untreated Arabidopsis, Arabidopsis exposed to uninfected male *M. quadrilineatus* or Arabidopsis exposed to AY-WB infected male *M. quadrilineatus*. Experiments were carried out in 3 controlled growth rooms with different conditions. Room A was set to 22°C consistently with a 10 hour day and 14 hour night. Room B was set for 24°C 16 hour day and 22°C 8 hour night and room C was 26°C 16 hour day and 22°C 8 hour night. Survival was quantified as the number of surviving females out of 8 after 4 day confinement to each plant. *D. maidis* survival was significantly increased on AY-WB infected Arabidopsis in rooms A and B but not in room C conditions  $F_{2,22} 5.364 P = 0.01^*$ ,  $F_{2,22} 3.67 P = 0.01^*$ , and  $F_{2,22} 11.482 P = 0.703$  respectively. In room C conditions insects had increased survival on untreated and uninfected Arabidopsis than in cooler conditions. Whist leafhoppers had higher survival on all plants in rooms B and C, female survival increase was significantly higher on uninfected inoculated Arabidopsis than on AY-WB infected plants in room C,  $F_{2,34} 9.364 P = 0.01^*$ . The experiment was repeated twice on 14-day-old leafhoppers. Four plant of each treatment were used in each condition per replicate. Bars show 2X standard error. Modeled by rep\*treatment\*condition using GLM with a binomial link function. Error bars shown are 2x standard error above and below means.



**Figure 3.2.1.4. *D. maidis* fecundity is further increased on AY-WB infected Arabidopsis in warmer conditions.**

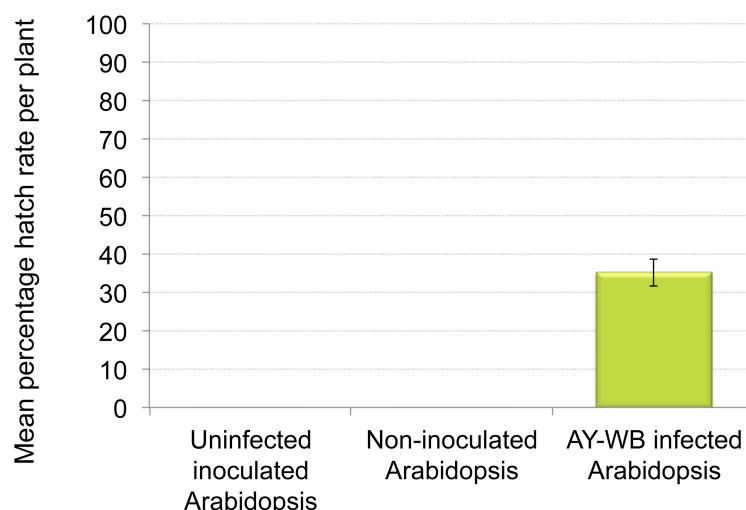
*D. maidis* fecundity was measured on untreated Arabidopsis, Arabidopsis exposed to uninfected male *M. quadrilineatus* or Arabidopsis exposed to AY-WB infected male *M. quadrilineatus*. Experiments were carried out in 3 controlled growth rooms with different conditions. Room A was set to 22°C consistently with a 10 hour day and 14 hour night. Room B was set for 24°C 16 hour day and 22°C 8 hour night and room C was 26°C 16 hour day and 22°C 8 hour night. Fecundity was quantified as the number of nymphs on plants 12-15 days after adults were removed. Eggs were counted 2 days after adult removal from each plant. *D. maidis* eggs and nymphs were only found on AY-WB infected Arabidopsis  $F_{2,14} 7.218 P = <0.001$  and  $F_{2,22} 20.932 P = <0.001$  respectively (A&B). No significant difference in egg or nymph numbers was seen on AY-WB infected Arabidopsis kept in different conditions  $F_{2,18} 14.921 P = 0.314$  and

$F_{2,17} = 8.457$   $P = 0.114$  respectively (A&B). The experiment was repeated twice on 14-day-old leafhoppers. Four plants of each treatment were used in each condition per replicate. Modeled by  $\text{rep} \times \text{treatment} \times \text{condition}$  using GLM with a binomial link function. Error bars shown are 2x standard error above and below means.



**Figure 3.2.1.5. *D. maidis* oviposited into mid veins of older *Arabidopsis* leaves**

*D. maidis* eggs were counted 2 days after adults were removed following 4-day confinement and oviposition. Most eggs were located under mature *Arabidopsis* leaves along the central primary vein. A few eggs were found on the topside of leaves but all were under epidermal cells of the mid vein (A). After oviposition the egg produced wax microfilaments that in many cases helped in locating eggs (A). To gain a closer view of eggs, I de-stained leaves with 100% ethanol over night and mounted leaves onto slides with 80% glycerol. Eggs were examined and photographed using a Nikon E800 fluorescence microscope (B). Eggs lost some wax microfilament in this treatment rendering the egg more visible (B). No eggs were found on uninfected *Arabidopsis* plants.

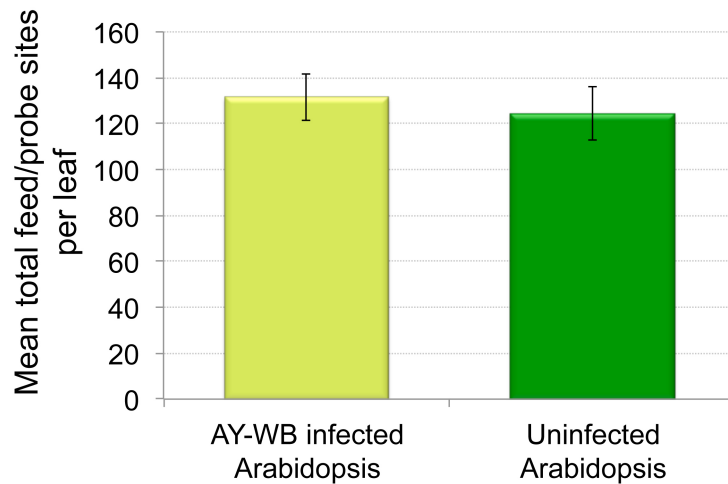


**Figure 3.2.1.6 A third of *D. maidis* eggs laid went on to hatch on AY-WB infected Arabidopsis**

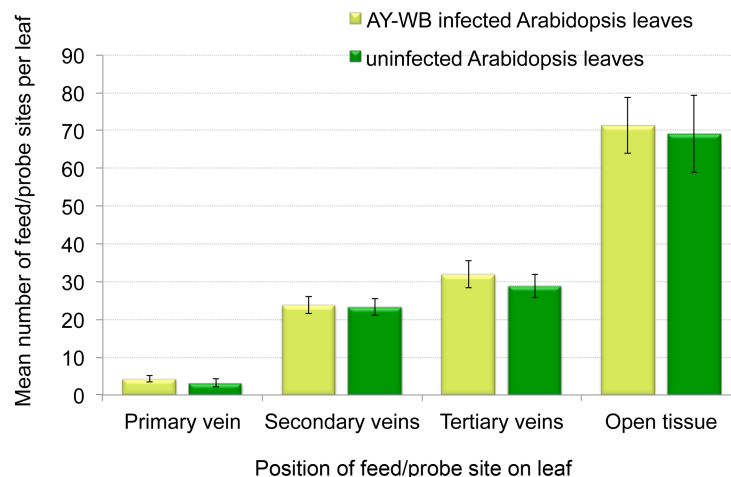
*D. maidis* eggs were counted 2 days after adults were removed following 4-day confinement and oviposition. Eggs were found under stems of mature Arabidopsis leaves and only on AY-WB infected plants. The percentage hatch rate was calculated by dividing the number of nymphs by the number eggs. Bars show 2X standard error.

### 3.2.2 *D. maidis* appears to feed more easily from AY-WB infected Arabidopsis.

To understand whether leafhopper feeding as well as oviposition was altered on AY-WB infected Arabidopsis, feeding sites of *D. maidis* were examined on healthy versus AY-WB infected Arabidopsis. Leafhoppers were exposed to single leaves for 5 days, then removed and leaves were stained with trypan blue stain to visualise dead tissue. This stain made feeding/probing sites visible in blue when leaves were mounted on slides (Fig. 3.2.2.3). The quantity of feeding sites was not altered on AY-WB infected Arabidopsis leaves (Fig. 3.2.2.1). Furthermore *D. maidis* fed predominantly on open tissue and tertiary veins of the leaves, this behaviour was unaltered between healthy and AY-WB infected leaves (Fig. 3.2.2.2).



**Figure 3.2.2.1. *D. maidis* feeding/probing level per leaf is unaltered on AY-WB infected Arabidopsis.** Five leafhoppers were confined to individual leaves for 5 days, and leaves were subsequently stained with trypan blue stain to highlight dead tissue around feed/probe sites. Treated leaves were mounted onto microscope slides and feed/probes sites were examined under a Nikon E800 fluorescent microscope. There was no significant difference in mean total feed/probe sites per leaf,  $F_{1,23} = 7.4236$   $P = 0.012$ . Modeled by rep\*treatment using GLM with a binomial link function. Error bars shown are 2x standard error above and below means.

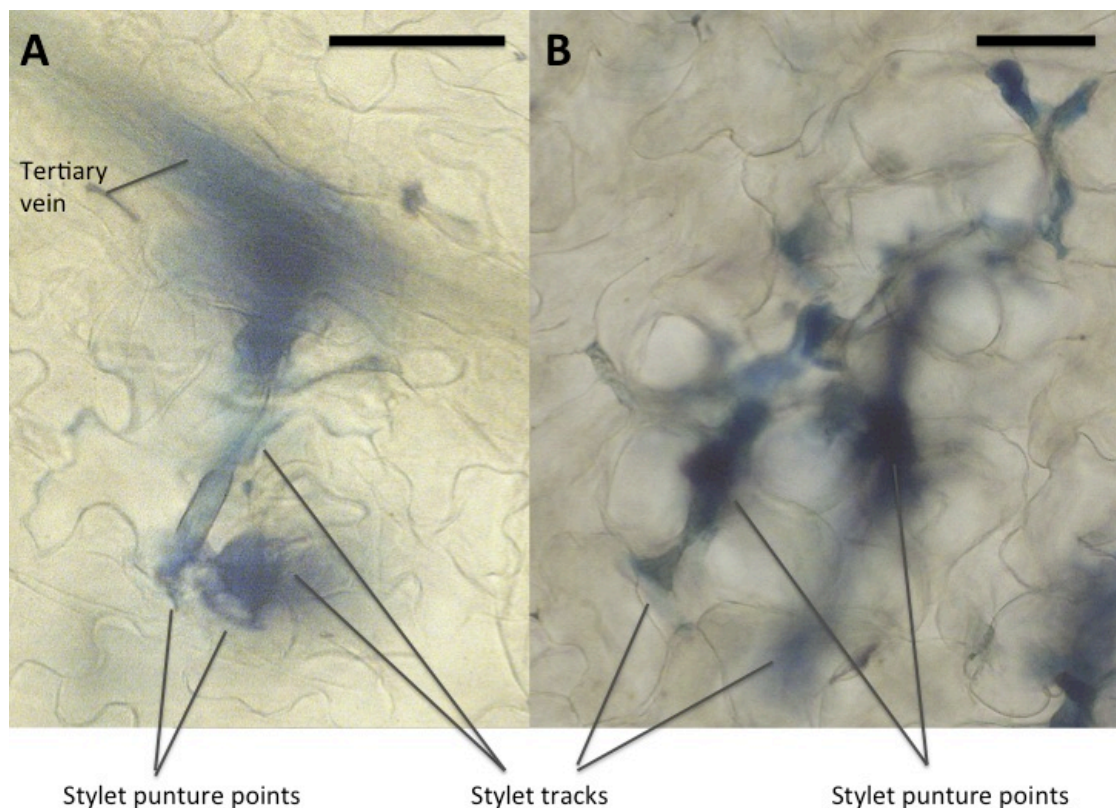


**Figure 3.2.2.2. *D. maidis* feeds from predominantly open tissue on Arabidopsis** Leaves exposed to leafhoppers and stained with trypan blue to highlight feed/probe sites were examined under a Nikon E800 fluorescent microscope. Feed/probe sites were counted on primary, secondary, tertiary veins, and open tissue of leaves. *D. maidis* concentrated feeding on the periphery of leaves and significantly more feed/probe sites were recorded on open tissue  $F_{4,18} = 2.8737$   $P = <0.001$ . No



significant difference was observed in feed/probe site locations between AY-WB infected leaves and uninfected leaves  $F_{1,22} = 287.0$   $P = 0.341$ . Modeled by  $\text{rep} \times \text{treatment} \times \text{location}$  using GLM with a poisson link function. Error bars shown are 2x standard error above and below means.

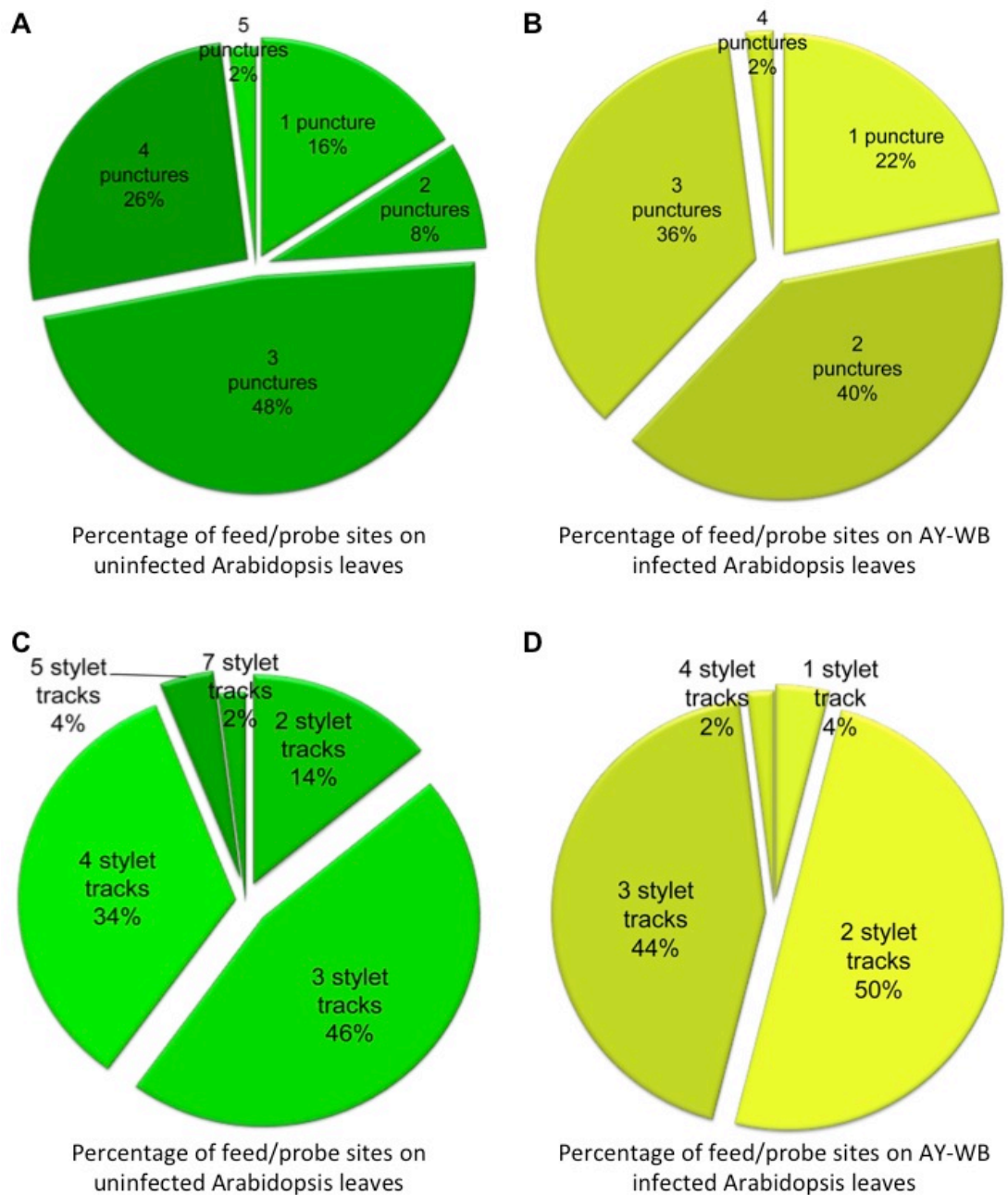
Interestingly, I found the physiology of feed/probe sites and number of stylet tracks from these sites were altered on AY-WB infected Arabidopsis leaves. *D. maidis* made more puncture points and more exploratory stylet tracks per feed/probe site on uninfected Arabidopsis leaves (Fig. 3.2.2.3). I examined the treated leaves under the microscope again, randomly selecting 10 circular quadrats per leaf, and recorded the number of stylet puncture points and the number of stylet tracks per feeding site. Puncture points over two epidermal cells apart were counted as separate feed/probe sites. I found that *D. maidis* feed/probe sites on uninfected leaves had 28% more exploratory stylet tracks per site and 25% more puncture points than sites on AY-WB infected leaves (Fig. 3.2.2.4).





**Figure 3.2.2.3. *D. maidis* feed/probe sites are clearer on AY-WB infected Arabidopsis leaves.**

Feed/probe sites were visualised on trypan blue stained leaves pre-exposed to leafhoppers for 5 days. Typical *D. maidis* feed/probe sites on AY-WB infected Arabidopsis leaves (A) are tidy with clear stylet puncture points and feeding tracks. On uninfected Arabidopsis leaves (B) *D. maidis* tends to puncture leaf surface more and create more stylet tracks. Images taken using a Nikon E800 fluorescent microscope and scale bars are 10 microns.

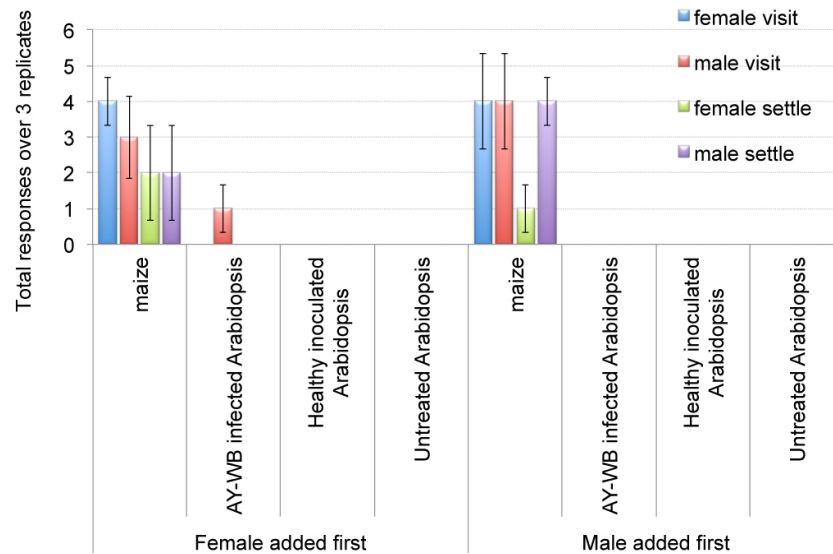


**Figure 3.2.2.4. *D. maidis* feed/probe sites have more puncture points and stylet tracks on uninfected Arabidopsis.**

*D. maidis* feed/probe sites were examined with a Nikon E800 fluorescent microscope and stylet tracks and puncture points were recorded. Feed/probe sites on AY-WB infected *Arabidopsis* had significantly less puncture points and less stylet tracks than uninfected *Arabidopsis*  $F_{1,36} = 8.203$   $P = 0.01$  and  $F_{1,24} = 4.103$   $P = 0.001$  respectively. On uninfected leaves 74% of feed/probe sites had 3 or 4 puncture points, 2x s.e.  $\pm 0.293$  (A), whereas 62% of sites on AY-WB infected leaves had 1 or 2 puncture points, 2x s.e.  $\pm 0.226$  (B). On AY-WB leaves, 94% of sites had 2 or 3 stylet tracks, 2x s.e.  $\pm 0.173$  (D), whereas 40% of uninfected sites had 4 or more stylet tracks, 2x s.e.  $\pm 0.260$  (C). Modeled by  $\text{rep} \times \text{treatment} \times \text{puncture points} \times \text{stylet tracks}$  using GLM with a poisson link function.

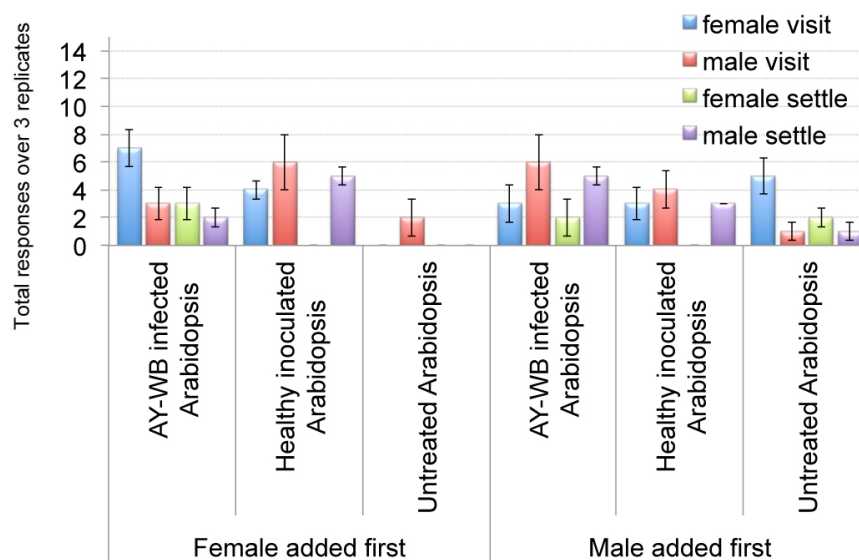
### 3.2.3 *D. maidis* shows no significant host choice preference on *Arabidopsis*.

To determine whether AY-WB infection in plants can affect *D. maidis* host plant choice, I conducted choice tests. Individual leafhoppers were added to a choice chamber with 4 7-week-old plants; a maize plant, an AY-WB infected *Arabidopsis*, an untreated *Arabidopsis*, and an *Arabidopsis* pre-inoculated with uninfected male *M. quadrilineatus*. Insects were introduced to the centre of the chamber and observed for 15 minutes. The first plant visit and the first settle to feed/probe were recorded for each insect. Chamber plants were used for two insect introductions before being replaced for fresh plants and the sex of the first insect was recorded to account for influence. In all experiments including maize, insects visited and settled on maize (Fig. 3.2.3.1). I repeated the experiments without maize and found there was no significant difference in first visit or feeding on any of the *Arabidopsis* plants (Fig. 3.2.3.2).



**Figure 3.2.3.1. *D. maidis* shows preference for host maize over Arabidopsis**

A choice chamber was used to assess whether AY-WB infection in Arabidopsis would make these plants attractive a host plant. Males and females showed significant preference for maize when maize plants were added into the cages,  $P = <0.001$  in all cases. Data analysed using contingency tables and Pearson chi square analysis in Genstat v.13.

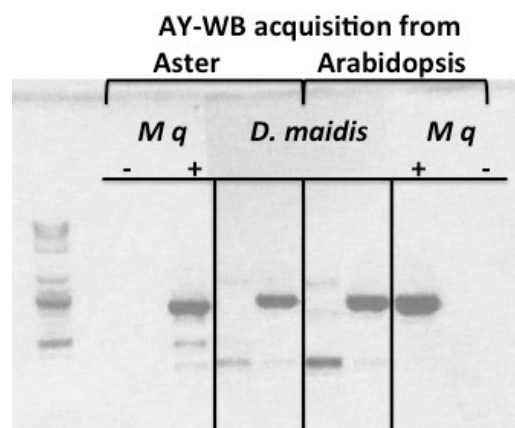


**Figure 3.2.3.2. *D. maidis* shows no preference AY-WB infected Arabidopsis**

Maize was removed from the choice experiments to assess AY-WB infection would render Arabidopsis more attractive to *D. maidis*. Male and female *D. maidis* exhibited no significant preference in visiting or settling on any Arabidopsis ( $P = >0.08$  in all cases). Data analysed using contingency tables and Pearson chi square analysis in Genstat v.13.

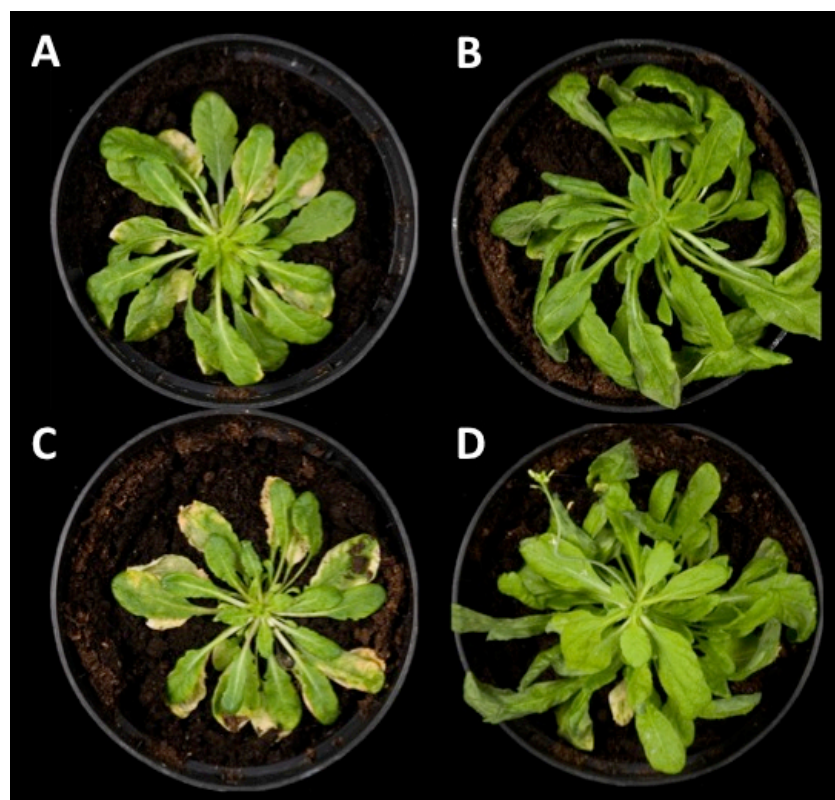
### 3.2.4 AY-WB infection in *D. maidis* has no effect on its survival or fecundity.

I confirmed that *D. maidis* can acquire AY-WB phytoplasmas from infected plants by exposing insects to AY-WB infected Arabidopsis and china aster for 10 days, then isolated DNA from individual leafhoppers and run a PCR screen. *D. maidis* is able to acquire AY-WB from infected china aster and Arabidopsis (Fig. 3.2.4.1). After 10day exposure to AY-WB infected Arabidopsis or Asters I enclosed leafhoppers on healthy china aster and Arabidopsis plants. No AY-WB symptoms developed on any test plants, and PCR analysis revealed no phytoplasma presence (Fig. 3.2.4.2 and Fig. 3.2.4.3). Kelly Bennett, an MSc student under my supervision, repeated these experiments with the addition of maize and lettuce and found *D. maidis* could acquire AY-WB from all infected plants but did not then pass AY-WB on to new plants (data not shown).



**Figure 3.2.4.1. *D. maidis* acquires AY-WB phytoplasma from infected aster and Arabidopsis**

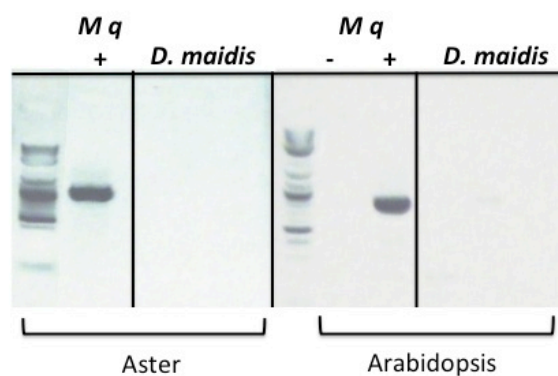
Leafhoppers were exposed to AY-WB infected Arabidopsis and aster for 10 days and assayed for phytoplasma presence using PCR. Post exposure DNA was extracted from single insects and PCR assays using AY-WB specific primers produced expected amplicons of 900bp size. Over all 32% of *D. maidis* insects tested positive for AY-WB.



**Figure 3.2.4.2. *D. maidis* is unable to transmit AY-WB to new plants**

20 AY-WB infected leafhoppers were added per plant to fresh 6-week-old Arabidopsis or asters. Of these plants, none exposed to AY-WB infected *D. maidis* developed symptoms of phytoplasma infection (examples B and D) after 3 weeks of confinement. AY-WB infected *M. quadrilineatus* were added to new aster and Arabidopsis plants as controls, all control plants exhibited strong symptoms (examples A and C) after 3 weeks confinement.

**AY-WB acquisition from AY-WB infected leafhoppers**

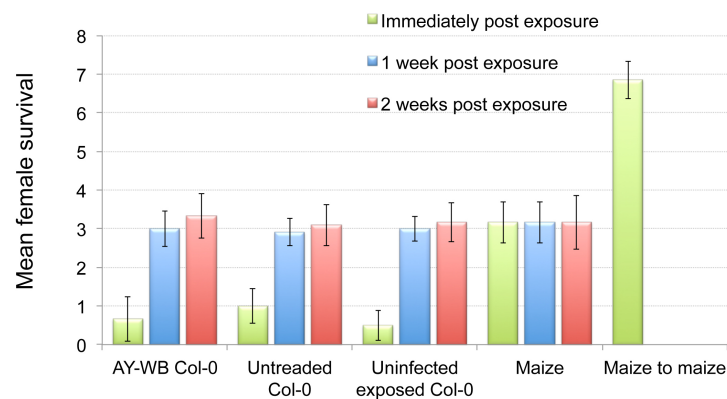


**Figure 3.2.4.3. Non-transmission of AY-WB by *D. maidis* confirmed by PCR**

Plants exposed to 20 AY-WB infected leafhoppers for 3 weeks were assayed for AY-WB phytoplasma presence with PCR. DNA was extracted from leaf tissue and PCR

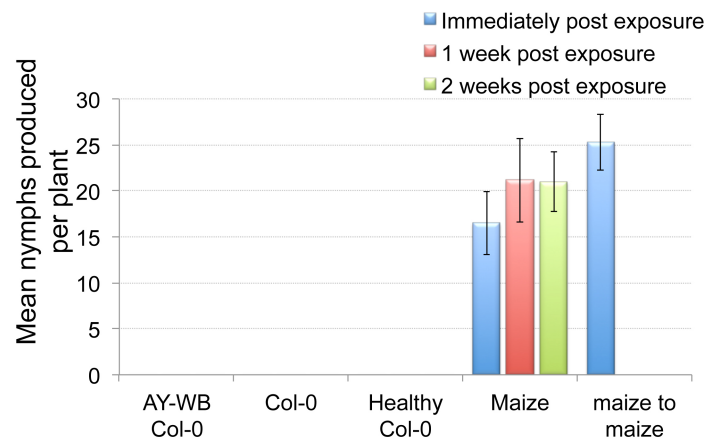
assays using AY-WB specific primers produced expected amplicons of 900bp size. All plants exposed to AY-WB infected *D. maidis* were negative for AY-WB. All plants exposed to AY-WB infected *M. quadrilineatus* tested positive for AY-WB.

*D. maidis* can acquire AY-WB phytoplasma but is unable to transmit it. To assess whether the phytoplasma inside the insect could affect survival or fecundity I tested *D. maidis* survival and fecundity at 3 different time points after exposure to AY-WB infected Arabidopsis. The leafhoppers were tested on untreated Arabidopsis, Arabidopsis exposed to male healthy *M. quadrilineatus*, and Arabidopsis exposed to AY-WB infected male *M. quadrilineatus*, and Maize. I found that *D. maidis* survival on Arabidopsis was unaffected by the presence of phytoplasma in the insects. Furthermore, *D. maidis* has high mortality immediately post exposure to AY-WB infected Arabidopsis when placed on further Arabidopsis plants (Fig. 3.2.4.4). AY-WB infected *D. maidis* showed lower survival on maize in comparison to *D. maidis* pre-exposed to maize (Fig 3.2.4.4). This indicates that AY-WB infection in the insect negatively affects *D. maidis* mortality. Fecundity was unaffected by AY-WB infection in *D. maidis*, no eggs or nymphs were found on any Arabidopsis plants (Fig. 3.2.4.5). In addition, no significant difference in fecundity was observed between *D. maidis* pre-exposed to maize and AY-WB infected *D. maidis* on host maize plants (Fig. 3.2.4.5).



**Figure 3.2.4.4. *D. maidis* survival on Arabidopsis in unaffected by AY-WB infection**

*D. maidis* were caged on AY-WB infected Arabidopsis for one week and then added to untreated Arabidopsis, uninfected inoculated Arabidopsis or AY-WB infected Arabidopsis at 3 different time points (immediately, after one week or after two weeks). *D. maidis* survival (number of surviving females out of 8 per plant after 4 days) was not significantly increased on any Arabidopsis plants  $F_{3,96} = 0.8748$   $P = 0.453$ . Duration of pause between AY-WB exposure and addition to test plant did prove significant, with insects showing high mortality immediately following exposure to AY-WB  $F_{2,96} = 19.45$   $P < 0.001$ . In addition AY-WB infected insects did show higher mortality on maize plants compared to *D. maidis* previously exposed to only maize  $F_{1,22} = 3.741$   $P < 0.001$ . Modeled by rep\*treatment\*time point using GLM with a binomial link function. Error bars shown are 2x standard error above and below means.



**Figure 3.2.4.5. AY-WB infection in *D. maidis* does not affect leafhopper fecundity on Arabidopsis**

Nymphs were only present on maize plants, thus post AY-WB exposure insects produced significantly more nymphs on maize  $F_{4,102} = 405.5278$   $P < 0.001$ . Duration of pause between AY-WB exposure and addition to test plant had no effect on nymph number found on maize plants  $F_{2,102} = 2.2135$   $P = 0.109$ . Furthermore, significantly more nymphs were produced on maize plants when insects had not been exposed to AY-WB  $F_{4,102} = 15.8462$   $P < 0.001$ . Modeled by rep\*treatment\*timepoint using GLM with a poisson link function. Error bars shown are 2x standard error above and below means.

## Discussion

### *3.3.1 AY-WB infection renders Arabidopsis a susceptible host to D. maidis.*

Survival of *D. maidis* on non-host *Arabidopsis* is low at 32% in females and 25% in males when enclosed on plants for 4 days. In contrast, *D. maidis* survival on AY-WB infected *Arabidopsis* increases to 54% and 48% for females and males respectively. In addition to the affect on survival, AY-WB infection in *Arabidopsis* also renders the plant suitable to *D. maidis* oviposition. Eggs and nymphs were only found on AY-WB infected *Arabidopsis*. I found *D. maidis* had low sporadic oviposition on AY-WB infected *Arabidopsis* and egg hatch was 35%. This hatch rate is similar to that found on non-host grasses when *D. maidis* is restricted to those non host plants (Pitre, 1970). This supports a hypothesis that when this leafhopper has access to only non-host plants it will try to feed and reproduce (Nault, 1994). It is unclear as to the cause of low hatch rate of eggs on non-host *Arabidopsis* plants. It is feasible that a cost may be endured in egg resistance to plant defences of the non-host plant. Similar costs have been recorded in other insects such as *drosophila* resistance to ectoparasites cost to egg hatch. Interestingly almost all eggs were found on the underside of mature *Arabidopsis* leaves along the lower mid vein. This location is hidden from potential egg parasitoids. On host maize plants eggs are oviposited along veins on the inside of leaf blades close to the main stem. This indicates the leafhoppers locate areas plants where their eggs are hidden.

### *3.3.2 AY-WB infection in Arabidopsis enables D. maidis to feed.*

*D. maidis* appeared to feed more easily on AY-WB infected *Arabidopsis* over uninfected. The over all quantity of feed/probe sites was not different but the damage to cells made at these sites were less pronounced on AY-WB infected *Arabidopsis*. Sites were predominantly on open tissue and tertiary veins of the leaves, but this behaviour was unaltered between healthy and AY-WB infected leaves. This is not surprising as the architecture of veins on maize leaves is considerably



different to that on *Arabidopsis* leaves. The location of *D. maidis* feed/probe sites was indicative of inefficient feeding on a non-familiar host. It is likely that AY-WB infection in *Arabidopsis* suppresses the plant response to *D. maidis* feeding, thus facilitating easier feeding of *D. maidis* on this non-host. Given enough time to adjust to this non-host, *D. maidis* may learn to feed more efficiently from the primary and secondary veins of leaves. *D. maidis* exhibited no significant preference for AY-WB infected plants over uninfected when given access to only *Arabidopsis*. Furthermore, in choice tests including maize, *D. maidis* visited and fed on maize in all tests. This demonstrates that whilst *D. maidis* would prefer to utilize its normal host, maize, if given no choice it is able to adapt to a new host. This adaption is aided by AY-WB infection in the plant.

### 3.3.3 *D. maidis* is unable to vector AY-WB.

*D. maidis* can acquire AY-WB phytoplasmas from infected *Arabidopsis* but they are unable to transmit them on to further plants. No symptoms developed and PCR analysis confirmed this. Furthermore, when *D. maidis* is a carrier of AY-WB, the survival and fecundity is unaffected by the presence of phytoplasmas in the insects. This suggests that while AY-WB can infect this non-host leafhopper, it is not able to overcome insect barriers of phytoplasma transmission. AY-WB may not be able to invade gut epithelial cells, basal lamina, plasmalemma, gut muscle cells, or invade the haemolymph. If AY-WB does get this far, the fat bodies in the haemolymph or the salivary glands may be an unbreachable barrier. Dissection, PCR and microscopy of insect tissues would be appropriate to assess where AY-WB gets in the insect. AY-WB phytoplasma affect on non-host insect-plant interactions, stems from the manipulation of the plant host rather than the insect host.

*3.3.4 D. maidis-Arabidopsis-AYWB is a useful model system in which to study phytoplasma manipulations of non-host interactions*

AY-WB manipulation of *D. maidis* survival and fecundity on non-host *Arabidopsis* provides a model system in which to investigate how phytoplasmas adapt to new potential vectors. AY-WB manipulation of *Arabidopsis* can be further studied using mutants with stable expression of AY-WB secreted effector proteins. Further research using these lines will yield clues as to how AY-WB and other AYPs modulate plants to become suitable host to non-host herbivorous insects such as *D. maidis*.

## **Chapter four: AY-WB secreted protein SAP11 plays differential roles in host and non-host manipulation**

### **Introduction**

#### *4.1.1 AY-WB infection manipulates insect and plant hosts and non-hosts.*

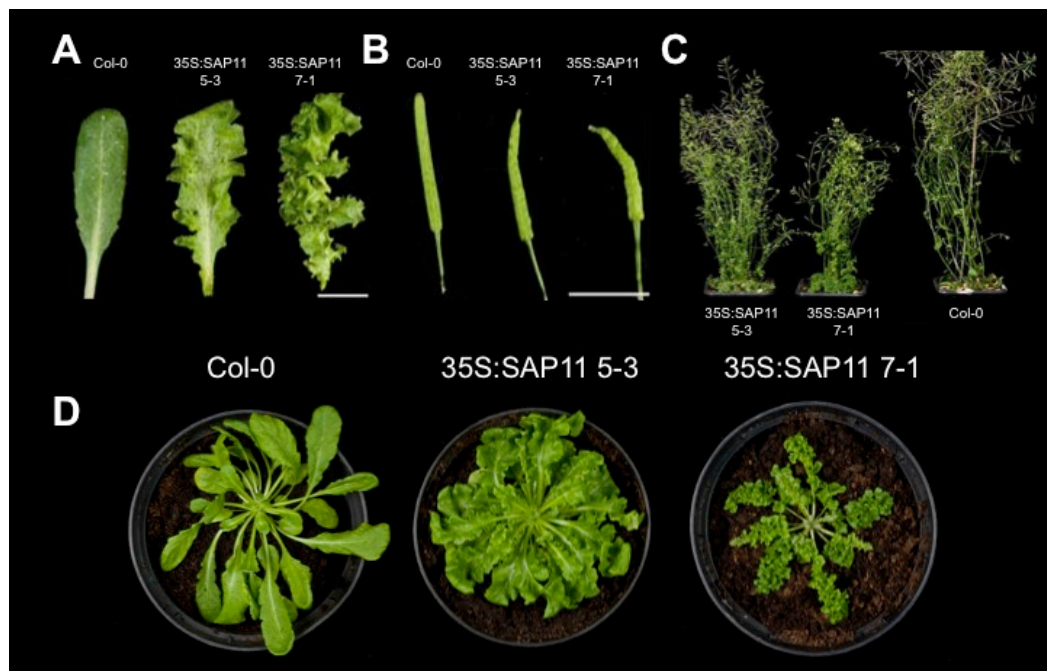
AY-WB phytoplasma vector leafhopper *Macrostelus quadrilineatus* has increased fecundity on AY-WB infected plants, including *Arabidopsis thaliana*. Infection within *Arabidopsis* generates proliferation of leafy tissue, stunting and green flowers. Increased fecundity is explained by an increase in oviposition by females exposed to infected plants (Chpt. 1). Furthermore, AY-WB infected females oviposit more on healthy plants than healthy females. However, no additive effect is observed when AY-WB infected insects are placed on AY-WB infected *Arabidopsis*. Interestingly, oviposition of AY-WB infected females is significantly increased only after 10 AY-WB acquisition. Indicating that it is unclear whether AY-WB is directly manipulating the insect to oviposit more or whether very early infection within the plant can influence leafhopper oviposition (Chpt. 2). In addition AY-WB infection in *Arabidopsis* renders the plant suitable for non-host maize specialist leafhopper *D. maidis*. Although *D. maidis* is unable to vector AY-WB, it is able to feed and reproduce on these plants and take up AY-WB with phloem whilst feeding (Chpt. 3). The effects of AY-WB infection in host and non-host plants are indicative of the evolution and successful transmission of phytoplasmas to crops worldwide.

#### *4.1.2 Of all the 56 AY-WB secreted proteins, SAP11 has the most profound effect on plant morphology*

AY-WB infection in plants generates dramatic changes in plant morphology such as proliferation of leaves, stems and branches (witches' brooms), phyllody, virescence, stunting or bolting, and proliferation of roots (Bertaccini, 2007). These morphological changes in the plant may attract insect vectors that preferentially lay eggs in

leaves, stems and some in roots (Hogenhout, *et al.*, 2008). AY-WB and other phytoplasmas probably use secreted effector proteins to induce morphological changes and down-regulate plant defence against the leafhopper vectors. Hogenhout and Loria, 2008, Hogenhout *et al.* 2008, Bai *et al.* 2006 and Bai *et al.* 2009). Phytoplasmas do not possess type three secretion systems but have functional SecA-dependant secretion systems from which effector proteins can be secreted (Kakizawa *et al.* 2001, Kakizawa *et al.* 2004).

The phytoplasma field isolate described by Beanland *et al.* as 'bolt' was isolated and named as AY-WB by Zhang *et al.* 2004 and fully sequenced by Bai *et al.* in 2006. The genome of AY-WB was mined for genes encoding candidate secreted effector proteins, of which 56 were described (Bai *et al.* 2009). To assess the function of each effector candidate, stable transgenic *Arabidopsis* lines were generated to express the effector candidate genes under control of the systemic *Cauliflower mosaic virus* 35S promoter. Three of these effector proteins induce clear morphological changes in *Arabidopsis* SAP11, SAP05 and SAP54 (Secreted Aster yellows Protein) (Sugio *et al.* 2011 and MacLean *et al.* 2011). SAP11 induces severe morphological changes within 35S:SAP11 *Arabidopsis* (Fig. 4.1.2.1), including crinkled leaves caused by cell proliferation, increased stems and branches and stunting of plants (Sugio *et al.* 2011).



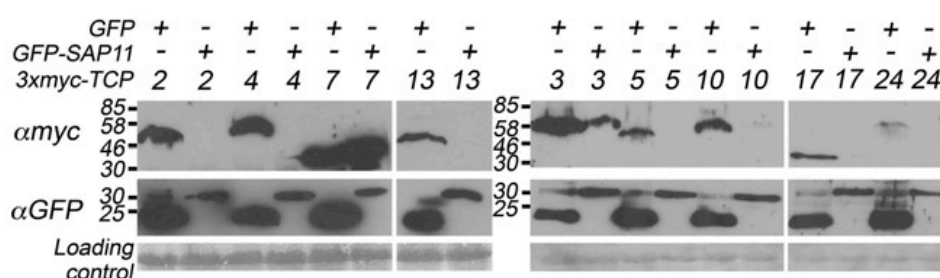
**Figure. 4.1.2.1. 35S:SAP11 Arabidopsis has severely altered morphology**

SAP11 induces crinkled leaves (A), siliques (B), significant proliferation of stems (C), and stunting of plants (C & D). Plants in D demonstrate the size of plants at the end of fecundity experiments; these plants are 9 weeks old. Figure modified from Sugio *et al.* 2011. Transgenic plants generated by A. Sugio, John Innes Centre, Norwich Research Park, Norwich.

#### *4.1.3 Secreted protein SAP11 destabilises TCP transcription factors affecting plant morphology and defence.*

SAP11 is known to target plant cell nuclei in transient expression assays and in AY-WB-infected plants (Bai *et al.* 2009, Sugio *et al.* 2011). This nuclear localisation enables that protein to generate changes in infected and transgenic plants (Sugio *et al.* 2011). A yeast two-hybrid screen identified (*CINCINNATA-TEOSINTE BRANCHED1*, *CYCLOIDIA*, *PCF*) *CIN*-related *TCP* transcription factors 2 and 13 as interactors with SAP11 (Sugio *et al.* 2011). *TCP* transcription factors are conserved among plants (Cubas *et al.* 1999, Howarth and Donoghue, 2006) and regulate many points of plant development (Palatnik *et al.* 2003, Schommer *et al.* 2008). Sugio *et al.* observed destabilisation of *CIN*-*TCP*s, in transient co-expression analyses (Fig. 4.1.2.2). This destabilisation of

CIN-TCPs results in the down-regulation of *LIPOXYGENASE2* (*LOX2*) expression and consequently the defence hormone jasmonic acid (JA) production, both of which play a crucial role in the Arabidopsis defence (Schrommer et al. 2008; Sugio et al. 2011). SAP11 may increase attraction of AY-WB infected plants by changing morphology of plants, but also by its' down-regulation of plant defences. This may allow vectors increased fitness on these plants.



**Figure. 4.1.3.1. SAP11 affects decreases the abundance of TCPs**

To investigate how SAP11 may affect the TCPs, transient co-expression analyses were conducted in *Nicotiana benthamiana* leaves of GFP-SAP11 and 3<sup>°</sup>— myc-TCPs under control of the 35S promoter. All CIN-TCPs were absent or decreased in abundance in the presence of GFP-SAP11 compared with GFP alone, whereas the abundance of class TCP7 was similar between the GFP-SAP11 and GFP treatments. Each experiment was repeated 9 times for TCP2, TCP4, TCP13, and TCP7 and twice for the other TCPs, and we constantly observed destabilization of CIN-TCPs but not TCP7. Figure taken from Sugio et al. 2011. Work completed by V. Grieve, John Innes Centre, Norwich Research Park, Norwich.

#### 4.1.4 Assessment of impact of SAP11 on leafhopper-plant interactions

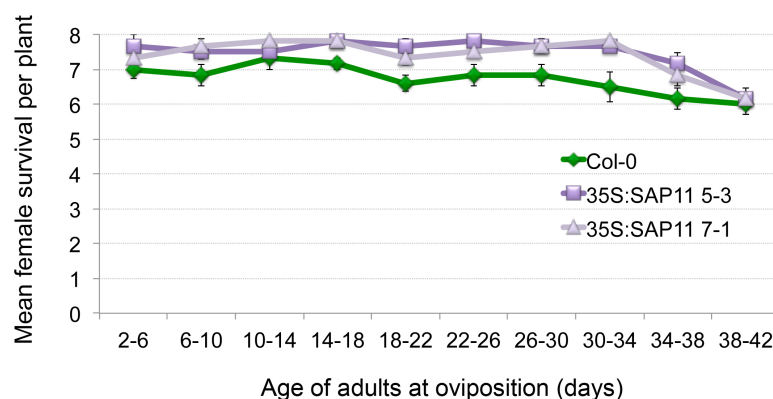
To understand the role of SAP11 in Arabidopsis encounters with *M. quadrilneatus*, I conducted survival and fecundity experiments on a number of Arabidopsis line expressing various forms of SAP11. In addition, I conducted fecundity trials on Arabidopsis with weakened defence response such as lines with reduced LOX2 and JA. Measuring the impact of SAP11 within Arabidopsis on leafhopper-plant interactions will allow more understanding of how this protein alone can influence the dispersal of phytoplasmas. I demonstrate here that

SAP11 affects host interaction *M. quadrilineatus* with *Arabidopsis* but has no effect on non-host *D. maidis* with *Arabidopsis* interaction. This highlights that whilst SAP11 is a powerful protein tool in the arsenal of AY-WB, other SAPs must play additional roles in host manipulation.

## Results

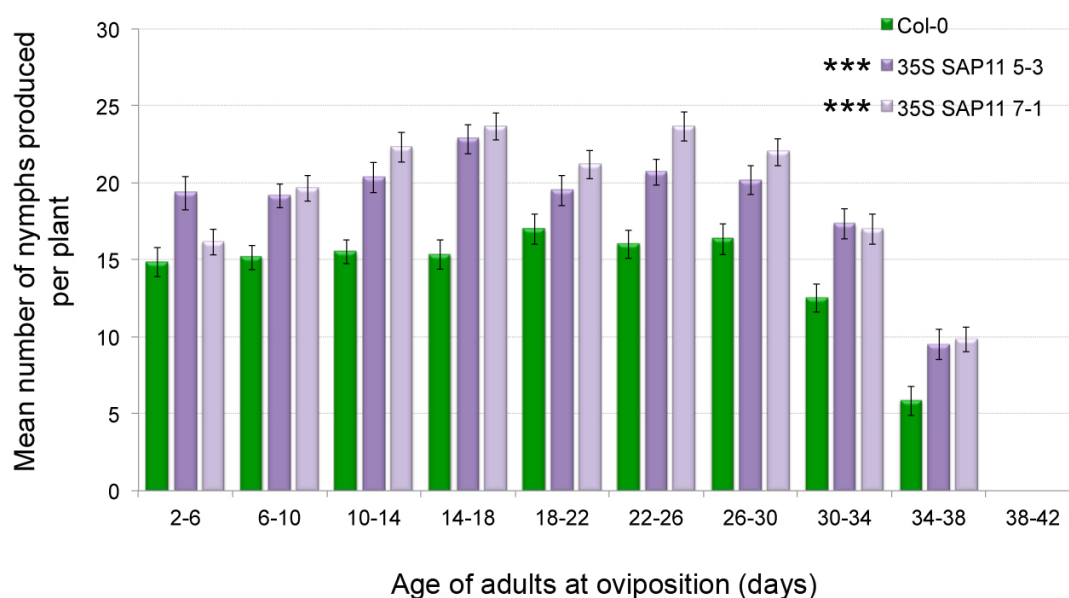
### 4.2.1 *M. quadrilineatus* has increased survival and fecundity on *Arabidopsis* with stable expression of SAP11.

SAP11 stable expression lines were used to test whether SAP11 expression alone could affect *M. quadrilineatus* survival fitness. I tested the survival and fecundity of *M. quadrilineatus* as a measure of fitness on *Arabidopsis thaliana* ecotype Col-0 as a control and 2 *Arabidopsis* lines with stable expression of SAP11, and 35S:SAP11 5-3 and 35S:SAP11 7-1. 8 females and 2 males of between 2 and 42 days in age were enclosed individually enclosed experimental plants in a time series experiment. All plants in experiments were 7 weeks old when insects were added. After 4 days on experimental plants, survival was counted. I found that survival was high on all plants no significant difference between Col-0 and SAP11 expression lines;  $F_{2,68} = 4.702$   $P = 0.625$  (Fig. 4.2.1). Eggs were counted during the 2 days following adults removal and oviposition was significantly increased on SAP11 lines;  $F_{2,106} = 76.09$ ,  $P < 0.001$  (Fig. 4.2.3). Fifteen days post adult removal plants were examined again for nymphs hatching from eggs previously counted. I found that in most cases eggs successfully hatched and nymph numbers were significantly increased on *Arabidopsis* with stable expression of SAP11 (Fig. 4.2.2). The percentage of successful nymph hatch was unchanged between control plants and SAP11 expression lines (Fig. 2.3.4). This indicates that oviposition increase is responsible for increased nymph production rather than hatch rate on infected plants.



**Figure. 4.2.1.1. Survival of *M. quadrilineatus* is unaffected by SAP11 expression in Arabidopsis**

Female survival was measured after 8 females were exposed to test plants for 4 days, surviving adults were counted on each plant. *M. quadrilineatus* survival was significantly increased on SAP11 lines of Arabidopsis over Col-0 controls,  $F_{2,118} = 18.69$ ,  $P < 0.001$ . Age of the insects had no significant effect on female survival,  $F_{9,118} = 4.287$ ,  $P = 0.106$ . The experiment included 10 leafhoppers per plant and 3 plants per treatment per time point. The whole experiment was repeated 3 times. Bars = 2 x standard error above and below predicted mean. Model analysed = constant\*replicate\*plant line\*age using GLM with a binomial link function.

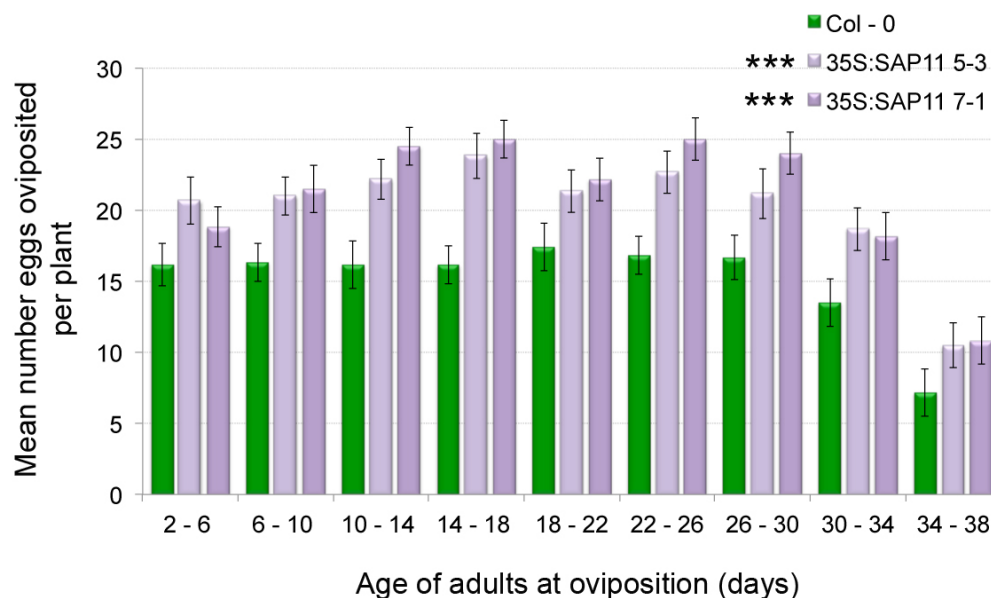


**Figure. 4.2.1.2 Nymph production of *M. quadrilineatus* is increased on SAP11 expression lines of Arabidopsis**

Numbers of nymphs were counted on each experimental plant 15 days following the removal of adults, to allow sufficient time for hatching and development to approximately 2<sup>nd</sup> instar. Nymph production significantly higher on the 35S::SAP11-5

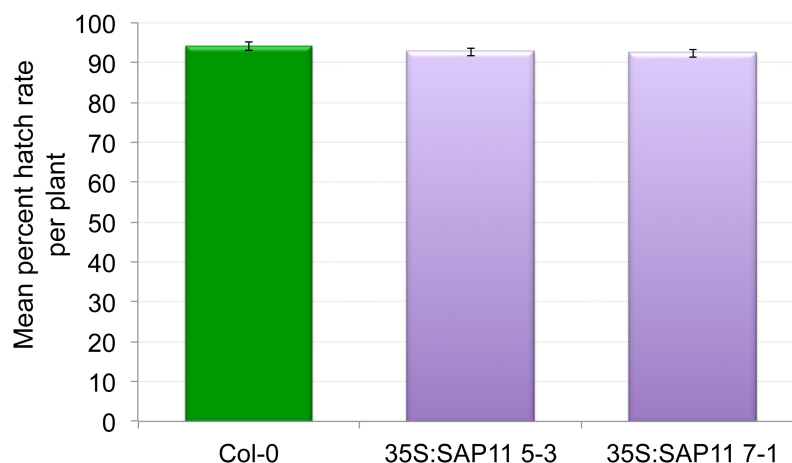


and 35S::*SAP11*-7 lines compared to Col-0 controls,  $F_{2,117} = 41.64$ ,  $P < 0.001$ . There was no significant difference in nymph numbers between the two 35S::*SAP11* lines  $F_{1,79} = 1.31$ ,  $P = 0.256$ . Female survival rates had no significant effect on leafhopper fecundity  $F_{1,117} = 1.3$ ,  $P = 0.256$ . Leafhopper age at oviposition did have a highly significant effect on nymph numbers  $F_{9,117} = 11.32$ ,  $P < 0.001$ . The experiment included 10 leafhoppers per plant and 3 plants per treatment per time point. The whole experiment was repeated 3 times. Bars = 2 x standard error above and below the predicted mean. Model analysed = constant\*replicate\*plant line\*age+female survival. Data analysed using a Poisson distribution and analysis of deviance in Genstat v13.



**Figure. 4.2.1.3. Oviposition of *M. quadrilineatus* is increased on *SAP11* expression lines of *Arabidopsis***

Two days after adult removal, leafhopper eggs were counted on plants. Leafhoppers oviposited significantly more eggs on the two 35S::*SAP11* lines;  $F_{2,106} = 76.09$ ,  $P < 0.001$ . Female survival did not have a significant effect on oviposition;  $F_{1,106} = 1.11$ ,  $P = 0.295$ . There was no significant difference in the egg numbers on the 35S::*SAP11* lines themselves,  $F_{1,79} = 13.412$ ,  $P = 0.233$ . The age of insects at oviposition had a highly significant effect on the numbers of eggs  $F_{9,106} = 12.768$ ,  $P = 0.001$ . The experiment included 10 leafhoppers per plant and 3 plants per treatment per time point. The whole experiment was repeated twice. Bars = 2 x standard error above and below predicted mean. Model analysed = constant\*replicate\*plant line\*age. Data analysed using a Poisson distribution and analysis of deviance in Genstat v13.

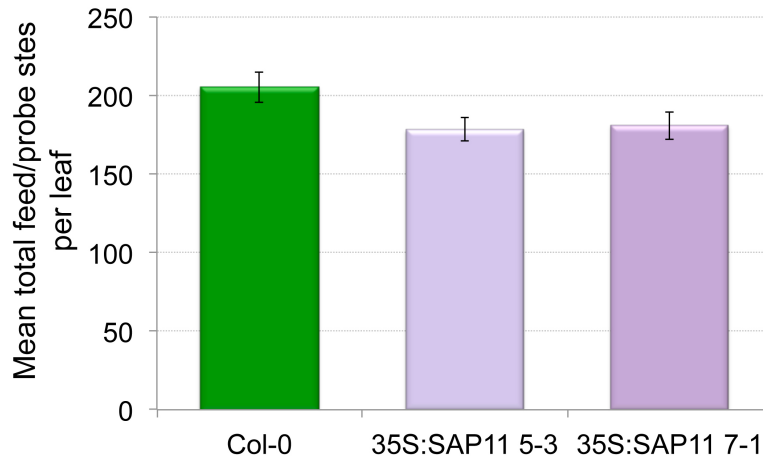


**Figure. 4.2.1.4. Percentage hatch rate of *M. quadrilineatus* unchanged by SAP11 expression in Arabidopsis**

The hatch rates of the eggs was calculated by dividing the number of eggs counted on experimental plants by the number of nymphs found 15 days later on plants. The hatch rates of eggs on the different plant lines were not significantly different;  $F_{2,97} = 0.89$ ,  $P = 0.414$ . Age also play no significant role in the hatch rates of eggs on any line  $F_{9,97} = 7.87$ ,  $P = 0.694$ . The experiment included 10 leafhoppers per plant and 3 plants per treatment per time point. The whole experiment was repeated twice. Model analysed = constant\*replicate\*plant line\*age using GLM with a poisson link function. Bars = 2x standard error above and below predicted means.

#### 4.2.2 *M. quadrilineatus* feeding is unaltered by 35S:SAP11 morphology

In addition, feeding of *M. quadrilineatus* was analysed by recording numbers of feed/probe sites on Col-0 versus SAP11 expression lines of Arabidopsis. The total quantity of feed/probe sites was reduced on SAP11 expression lines (Fig. 4.2.2.1). Overall feed/probe site location was unchanged on SAP11 expression lines compared to Col-0 (Fig. 4.2.2.2). Furthermore, no significant difference in feed/probe behaviour, in terms of numbers of stylet tracks per puncture points, was recorded. The vast majority of feed/probe sites consisted of just one puncture point and one stylet track towards phloem (Fig. 4.2.2.3).

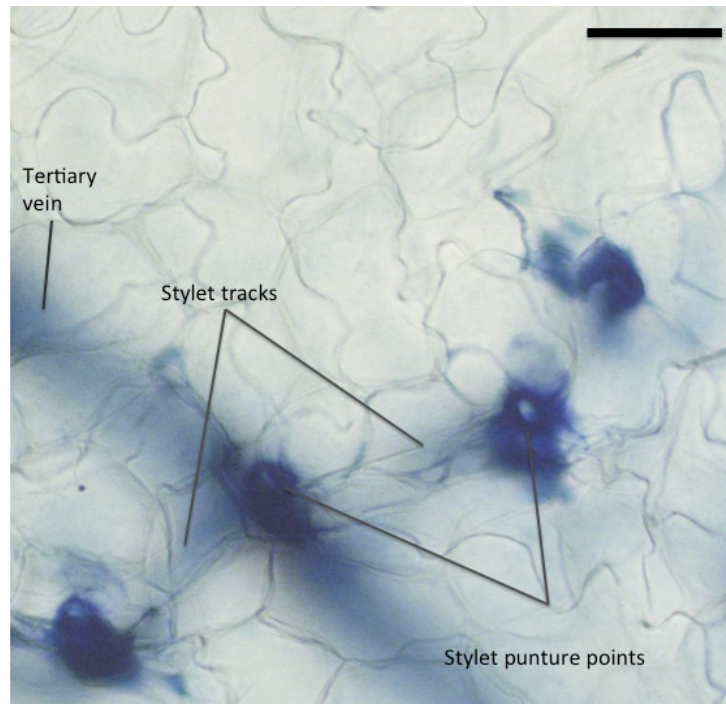


**Figure. 4.2.2.1. Number of *M. quadrilineatus* feed/probe sites per leaf was reduced by SAP11 expression in Arabidopsis**

*M. quadrilineatus* leafhoppers feed/probe sites were determined as visible puncture points and stylet tracks between plant cells to phloem. Mature single leaves of 4-week old Arabidopsis lines were enclosed with 5 adult *M. quadrilineatus* in 15ml falcone tube cages. After 5 days exposure, leaves were harvested and subjected to trypan blue staining to visualize the feed/probe sites, then recorded using fluorescence microscopy. The numbers of feed/prob sites were reduced significantly on the two 35S:: *SAP11* lines compared to Col-0 controls,  $F_{2, 25} = 14.1$ ,  $P < 0.001$ . Twelve Col-0 leaves and 8 leaves of each of the two 35S::*SAP11* lines were analyzed. Model analysed = constant\*plant line using GLM with a poisson link function. Bars = 2x standard error above and below predicted means.

<b>A</b>	Primary vein	Secondary vein	Tertiary vein	Open tissue
Col-0	118.42	43.17	26.75	16.92
S.E.	+/-3.72	+/-2.63	+/-1.98	+/-2.38
35S:SAP11 5-3	97	42.88	23.25	15.25
S.E.	+/-2.11	+/-1.63	+/-2.14	+/-2.21
35S:SAP11 7-1	102.63	40.75	23.25	14.25
S.E.	+/-3.12	+/-1.94	+/-2.11	+/-2.71

**B**

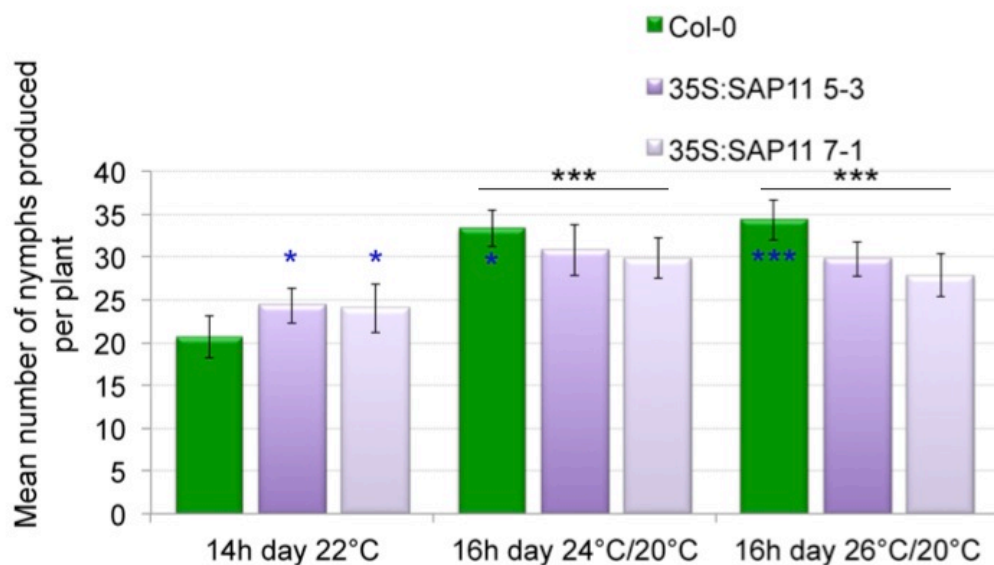


**Figure. 4.2.2.2 Location and structure of *M. quadrilineatus* feed/probe sites on leaves is unchanged by SAP11 expression in Arabidopsis.** Feed/probe sites on Arabidopsis leaf veins and open tissue were counted to ascertain any differences in location of feeding between Arabidopsis lines (A). The 'damage' caused by feed/probe sites (number of stylet tracks per puncture points) was also recorded as an indication of ease of leafhopper feeding (B). Location of feed/probe sites were not significantly different on any plant lines,  $F_{2,29} = 14.037$   $P = 0.09$  **A**. SAP11 had no significant effect on the 'damage' caused at feed/probe sites of *M. quadrilineatus*,  $F_{2,32} = 1.824$   $P = 0.912$ . Typical feed/probe sites of 1 stylet track per puncture point are highlighted in **B**. The experiment included 5 leafhoppers per leaf for 5 days, 6 leaves per treatment and the whole experiment was repeated 3 times. The crinkled morphology of the 35S::SAP11 plant leaves did not alter leafhopper-feeding sites **A**. Models analysed = **A** constant\*plant line\*location, and **B** constant\*plant line\*puncture points\* stylet tracks using GLM with a poisson link function.

#### *4.2.3 SAP11 expression in Arabidopsis does not induce higher vector fitness in warmer climate conditions.*

Survival and fecundity experiments were repeated in conditions with higher temperatures and increased day length, which are more

favourable to *M. quadrilineatus* leafhoppers. *M. quadrilineatus* exhibited similar survival on control Col-0 Arabidopsis to Arabidopsis with SAP11 expression (Fig. 4.2.3.1). From data gained in controlled growth rooms set at 22°C and 14hour days, *M. quadrilineatus* fecundity is significantly affected by SAP11 expression in Arabidopsis (Fig. 4.2.3.2). Here the aim was to ascertain whether increase in temperature and day length have impacts on the effect of SAP11 on *M. quadrilineatus* fecundity. Three growth room conditions were used, 22°C 14h/10hr day/night, 24°C/20°C 16h/8h day/night, and 26°C/20°C 16h/8h day/night. *M. quadrilineatus* showed a highly significant increase in fecundity in warmer conditions with longer day length on all plant lines (black asterisks in Fig.4.2.3.1). In addition, *M. quadrilineatus* fecundity was significantly higher on 35S::SAP11 Arabidopsis only in 22°C 14h/10hr day/night conditions. In warmer conditions of 24°C/20°C 16h/8h day/night, and 26°C/20°C 16h/8h day/night, fecundity was significantly higher on Col-0 controls than 35S::SAP11 lines (blue asterisks in Fig. 4.2.3.1).



**Figure 4.2.3.1. SAP11 expression in Arabidopsis does not induce higher vector fitness in warmer climate conditions**

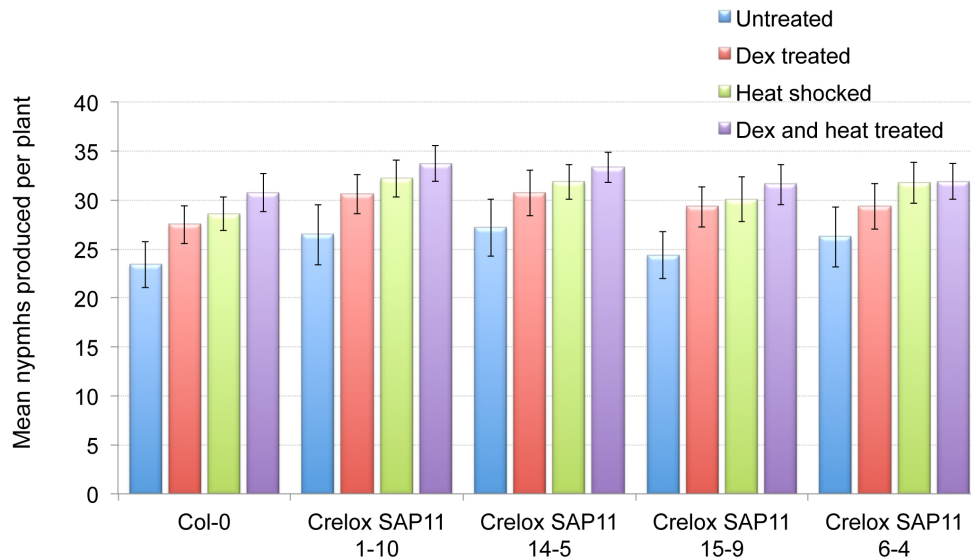
In order to assess the impact of SAP11 expression together with temperature increase on *M. quadrilineatus* fecundity, assays were completed in three controlled growth rooms, 22°C 14h/10hr day/night, 24°C/20°C 16h/8h day/night, and 26°C/20°C

16h/8h day/night ('conditions' in model). *M. quadrilineatus* exhibited a highly significant increase in fecundity in warmer conditions with longer day length on 35S::SAP11 and Col-0 controls \*\*\*F<sub>2,32</sub> = 11.847 P = 0.001. Fecundity was significantly higher on 35S::SAP11 Arabidopsis but only in 22°C 14h/10hr day/night conditions \*F<sub>2,24</sub> = 3.479 P = 0.041. *M. quadrilineatus* fecundity was significantly increased on Col-0 controls in 24°C/20°C 16h/8h day/night, and 26°C/20°C 16h/8h day/night conditions than 35S::SAP11 lines, \*F<sub>1,21</sub> = 8.734 P = 0.024 and \*\*\*F<sub>1,21</sub> = 12.673 P = 0.001 respectively. This indicates that SAP11 accumulation in Arabidopsis has an impact *M. quadrilineatus* fecundity but that an increase in temperature and day length may cancel out this effect, or even compromise the leafhopper fecundity. Experiment included 10 leafhoppers per plant and 3 plants per treatment per room. Model analysed = constant\*condition\*plant line using GLM with a poisson link function. Bars = 2x standard error above and below predicted means.

#### 4.2.4 Inducible SAP11 proves inconclusive due to induction treatment

SAP11 accumulation in Arabidopsis has a significant increasing effect on *M. quadrilineatus* fecundity (Fig. 4.2.1.2). Arabidopsis lines with inducible SAP11 using the CreLox system were used to assess whether fecundity also is increased after induction of SAP11. Using the CreLox system to induce Arabidopsis require treating plants with Dex and heat shock to initiate the CreLox system and SAP11 expression. These treatments alone and cumulatively had potential to alter plant fitness and fecundity and so were carefully controlled for. Four lines of the CreLox SAP11 were used with 4 treatments to control for the impact of each step in the induction process on Arabidopsis. Treatments were untreated, Dex treated, heat shock, and Dex+heat shock. Fecundity was examine on plants at 4 timepoints post-treatment, 1 day, 4 days, 7 days and 10 days, thus accounting for SAP11 accumulation in Arabidopsis. Whilst timepoint post-infection and plant line had significant effects on fecundity, increases in control plants presented an issue in testing the hypothesis. *M. quadrilineatus* fecundity was significantly increased on all plants treated with Dex or heat shock or Dex+heat shock (Fig. 4.2.4.1). *M. quadrilineatus* fecundity was not significantly higher on SAP11 induced lines compared with lines merely treated with Dex or heat shocked. Thus, any effect that induction and accumulation of

SAP11 in the Arabidopsis lines may have, was obscured by the effect seen in Dex, heat shocked, and Dex+heat shocked treated plants.



**Figure. 4.2.4.1. Treatment masks affect of inducible SAP11 on *M. quadrilineatus* fecundity**

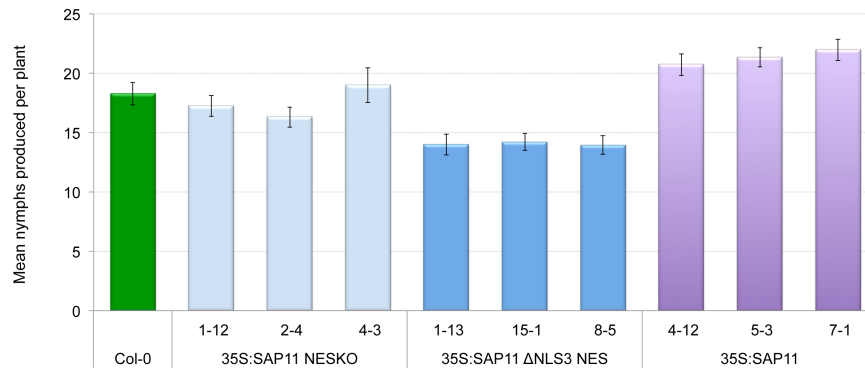
*M. quadrilineatus* fecundity is increased on Arabidopsis with stable system accumulation of SAP11. Arabidopsis lines with inducible SAP11 using the CreLox system were used to assess whether *M. quadrilineatus* fecundity would increase in response to induction of SAP11 accumulation in the plant. Four lines of the CreLox SAP11 Arabidopsis were used with 4 treatments to control for the impact of each step in the induction process on Arabidopsis. Insect fecundity was studied at 4 different time points after treatments. Three plants, per 4 treatments, per 5 plant lines, per 4 post-treatment timepoints were examined in each experiment and the whole experiment was repeated 3 times (thus 240 plants and 2400 leafhoppers). The timepoint post-treatment had a highly significant effect on fecundity,  $F_{3,320} = 286.3$ ,  $P = <0.001$  (means of all timepoints per treatment per line are shown). *M. quadrilineatus* fecundity was also significantly affected by the plant line  $F_{4,320} = 24.252$ ,  $P = <0.001$ . However, there was no significant effect on fecundity of insects at different timepoints post-treatment on the 5 different plant lines  $F_{9,320} = 34.1667$ ,  $P = 0.998$ . There was also no significant impact on fecundity of insects on the 5 different plant lines undergoing the 4 different treatments  $F_{12,320} = 2.4582$ ,  $P = 0.943$ . Plant treatment alone had a highly significant effect on fecundity  $F_{3,320} = 104.5723$ ,  $P = <0.001$ . To investigate this further Dex, Heat and Dex+Heat treatments were modeled individually with untreated controls to examine their effects. *M. quadrilineatus*

fecundity was significantly increased on Arabidopsis lines treated with Dex alone, heat shock alone and when treated with Dex and heat shocked,  $F_{1,119} = 6.7834$ ,  $P = 0.009$ ,  $F_{1,119} = 14.5622$ ,  $P = <0.001$ , and  $F_{1,119} = 16.44$ ,  $P = <0.001$  respectively. These results are strong evidence that any affect of SAP11 induction on insect fecundity is masked by the treatments to induce SAP11 in the plants. Modeled by rep\*treatment\*plant line\*age using GLM with a poisson link function. Bars = 2x standard error above and below predicted means.

#### 4.2.5 *SAP11 with no nuclear localization has a negative effect on M. quadrilineatus*

Arabidopsis lines with stable expression of SAP11 with nuclear export signal knock out 35S:*SAP11 NESKO* and an impaired nuclear localization signal and intact nuclear export signal 35S:*SAP11 ΔNLS3 NES* were used to assess the impact of 'faulty' SAP11 on leafhopper–plant interactions. Accumulated SAP11 in Arabidopsis has a significantly positive effect on *M. quadrilineatus* fecundity, however, when combined with warmer temperatures and longer day lengths, a negative effect is observed. Here, compromised nuclear localization has a negative impact on *M. quadrilineatus* fecundity. *M. quadrilineatus* fecundity on significantly lower on 35S:*SAP11 ΔNLS3 NES* compared to Col-0, and that there is no significant difference between Col-0 and 35S:*SAP11 NESKO* (Fig. 4.2.5.1). It is probable that a 'fault' is SAP11 nuclear localization renders the protein redundant in affecting *M. quadrilineatus* fecundity. In addition, it indicates that accumulation of faulty SAP11 may induce responses in the plant that have a negative effect on *M. quadrilineatus* oviposition.





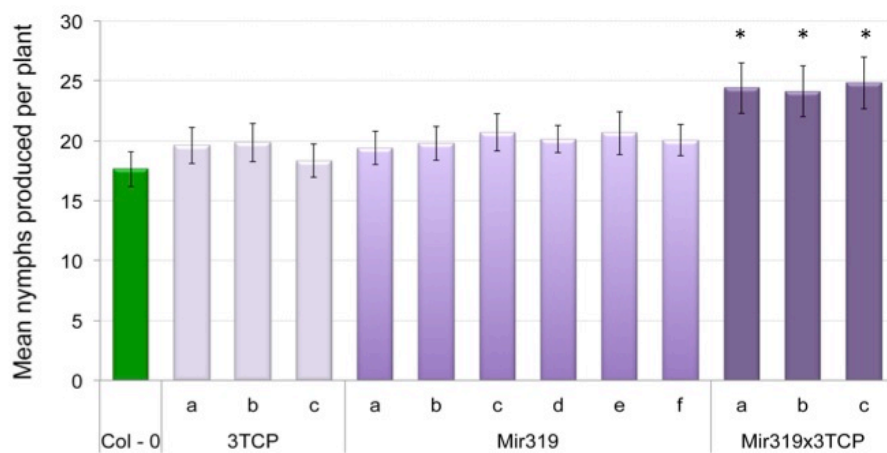
**Figure. 4.2.5.1. Compromised nuclear localization renders SAP11 useless in manipulating *M. quadrilineatus* fecundity**

Fecundity was examined on Arabidopsis with stable expression of SAP11 with compromised nuclear localization signals. This was to assess whether this function of SAP11 was key to the efficacy of SAP11 in increasing vector fecundity on Arabidopsis. Three lines of 35S: *SAP11 NESKO*, and 35S:*SAP11 ΔNLS3 NES* were compared to 3 lines of 35S::SAP11 and Col-0 control plants. Three plants per plant line were examined at 4 leafhopper age points and the whole experiment was repeated 3 times (360 plants and 3600 insects). *M. quadrilineatus* fecundity on significantly lower on 35S:*SAP11 ΔNLS3 NES* compared to Col-0,  $F_{1,127} = 26.857$ ,  $P = <0.001$ . There was no significant difference between fecundity on Col-0 control plants and 35S: *SAP11 NESKO*,  $F_{1,122} = 16.6952$ ,  $P = 0.097$ . Fecundity on SAP11 controls 35S::SAP11 was significantly higher than on Col-0 and 35S: *SAP11 NESKO*, and 35S:*SAP11 ΔNLS3 NES* lines  $F_{9,238} = 38.4576$ ,  $P = <0.001$ . Age of leafhoppers at oviposition had a significant effect on fecundity,  $F_{3,238} = 5.677$ ,  $P = <0.001$ . These data suggest that SAP11 nuclear localization is an important contributor to the effect that SAP11 has on *M. quadrilineatus* fecundity. In addition, it is plausible that accumulation of 'faulty' SAP11 has an effect on the plant, which translates to a negative effect on leafhopper fecundity. Modeled by rep\*treatment\*plant line\*age using GLM with a poisson link function. Bars = 2x standard error above and below predicted means.

#### 4.2.6 Assessment of destabilised TCPs on leafhopper fecundity

A yeast two-hybrid screen identified (*CINCINNATA-TEOSINTE BRANCHED1*, *CYCLOIDIA*, *PCF*) *CIN*-related TCP transcription factors 2 and 13 as interactors with SAP11 (Sugio *et al.* 2011). TCP transcription factors regulate many points of plant development (Palatnik *et al.* 2003,

Schommer et al, 2008). Sugio *et al.* observed destabilisation of CIN-TCPs, in transient co-expression analyses (Fig. 4.1.2.2). Destabilisation of these transcription factors results in the down-regulation of *LIPOXYGENASE2* (*LOX2*) expression and consequently the defence hormone jasmonic acid (JA) (Schommer *et al.* 2008; Sugio *et al.* 2011). Co-expression analyses and indicated that SAP11 destabilises all the Class II CIN-TCPs, but not Class I TCP7. As SAP11 accumulation in *Arabidopsis* has such a significant effect on *M. quadrilineatus* fecundity, it is possible that the destabilisation of these TCPs may also have an effect on *M. quadrilineatus* fecundity. To assess whether destabilisation of TCPs affects leafhopper fecundity existing *miR319a*, *miR-3TCP* and the octuple *cin-tcp miR319a x miR-3TCP* *Arabidopsis* lines were used in fecundity assays. Line *miR-3TCP* negatively regulates expression of TCP5, 13, and 17, in *miR319a* TCP2, 3, 4, 10 and 24 are negatively regulated, and *miR319a x miR-3TCP* negatively regulates all eight of these TCPs. The *miR-3TCP* lines have less obvious deeply lobed and highly crinkled leaf morphology compared to *miR319a* and the octuple *cin-tcp* lines. Fecundity is only significantly increased on the octuple *cin-tcp miR319a x miR-3TCP* lines (Fig. 4.2.6.1). Indicating that only when all 8 TCPs are compromised, is fecundity increased.



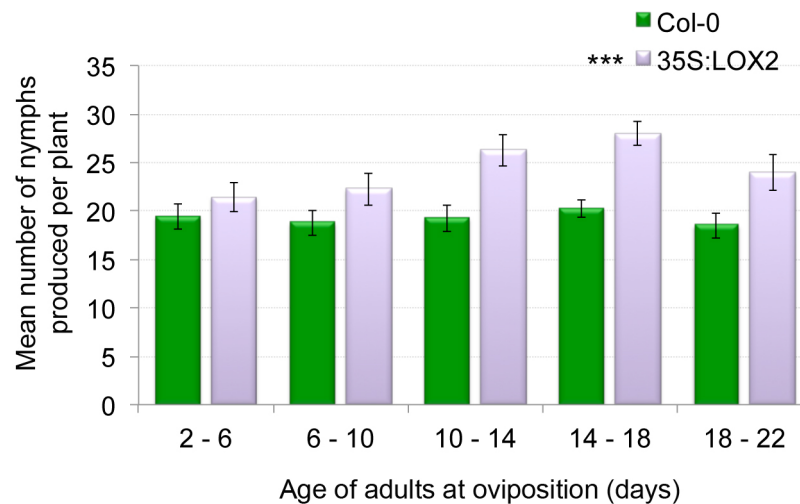
**Figure. 4.2.6.1. Destabilisation of TCPs increases *M. quadrilineatus* fecundity**

Arabidopsis lines *miR319a*, *miR-3TCP* and the octuple *cin-tcp miR319a x miR-3TCP* were used in fecundity assays to examine *M. quadrilineatus* fecundity on Arabidopsis with compromised TCPs. Line *miR-3TCP* negatively regulates expression of TCP5, 13, and 17, in *miR319a* TCP2, 3, 4, 10 and 24 are negatively regulated, and *miR319a x miR-3TCP* negatively regulates all eight of these TCPs. Fecundity was not significantly increased on *miR319a* and *miR-3TCP* lines compared with control Col-0,  $F_{8,184} = 34.847$ ,  $P = 0.086$ . When lines were individually compared with Col-0, fecundity was significantly increased on *miR319a* lines c and e  $F_{1,23} = 3.9435$ ,  $P = 0.01$  and  $F_{1,21} = 7.548$ ,  $P = 0.001$  respectively. A highly significant increase in fecundity was found on the octuple *cin-tcp miR319a x miR-3TCP* lines  $F_{12,184} = 46.5734$ ,  $P = <0.001$ . Female age at oviposition also had a significant effect on fecundity,  $F_{3,184} = 13.7433$ ,  $P = <0.001$  (Mean nymph production of all age groups per line are shown). The experiment included 3 plants per plant line at 4 leafhopper age points and the whole experiment was repeated 3 times (468 plants and 4680 leafhoppers). Modeled by rep\*plant line\*age using GLM with a poisson link function. Bars = 2x standard error above and below means.

**4.2.7 *M. quadrilineatus* shows increased fitness when *SAP11* downstream targets are compromised in Arabidopsis**

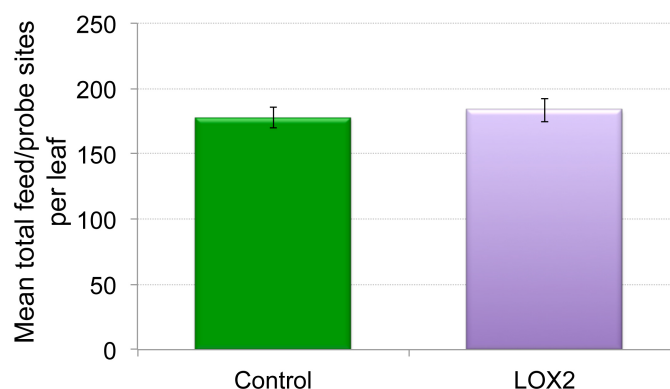
Vector leafhopper *M. quadrilineatus* has increased fecundity on Arabidopsis that has AY-WB secreted protein *SAP11* stably expressed. *SAP11* targets TCPs in Arabidopsis and these in-turn reduce the production of *LIPOXYGENASE2* (*LOX2*) and consequently the defence hormone jasmonic acid (JA) in Arabidopsis. CIN-TCPs positively regulate plant senescence through the upregulation of *LOX2* by TCP4-binding to *LOX2* promoter sites. *LOX2* encodes a lipoxygenase that mediates the first step of jasmonic acid (JA) synthesis pathway. Sugio *et al.* found that the expression levels of *LOX2* were considerably lower in the 35S::*SAP11* lines versus wild type Col-0 produce less JA upon wounding than wild-type Col-0 (Sugio *et al.* 2011). *M. quadrilineatus* fecundity is increased on Arabidopsis with down-regulation of 8 TCPs (Fig. 4.2.7.1). To assess whether *M. quadrilineatus* fitness is also affected by compromise of down stream targets of *SAP11*, I conducted survival and fecundity assays on Arabidopsis with silenced lipoxygenase 2 (*LOX2*) and reduced synthesis of JA (*Jar1-1*). *M.*

*quadrilineatus* survival was high on all lines but fecundity is significantly increased on LOX2 and Jar1-1 Arabidopsis (Fig 4.2.7.2 and Fig. 4.2.7.3).



**Figure. 4.2.7.1. *M. quadrilineatus* fecundity is increased on Arabidopsis with reduced expression of LOX2**

Assays were carried out on LOX2 deficient Arabidopsis to assess whether the compromise of down stream targets of SAP11, also has an effect on *M. quadrilineatus* fecundity. Eight female and 2 male *M. quadrilineatus* were added to each experimental plant for 4 days. Numbers of nymphs were counted 15 days after adults were removed from plants to allow them to develop to at least 2<sup>nd</sup> instar. The experiment included 3 plants of each plant line per 5 leafhopper age ranges and the whole experiment was repeated 3 times (90 plants and 900 leafhoppers). *M. quadrilineatus* fecundity was significantly increased on LOX2 silenced Arabidopsis compared with Col-0  $F_{1,64} = 27.485$ ,  $P = <0.001$ . Age of leafhoppers at oviposition also had a significant increasing effect  $F_{4,64} = 3.4355$ ,  $P = 0.001$ . Modeled by rep\*plant line\*age using GLM with a poisson link function. Bars = 2x standard error above and below means.

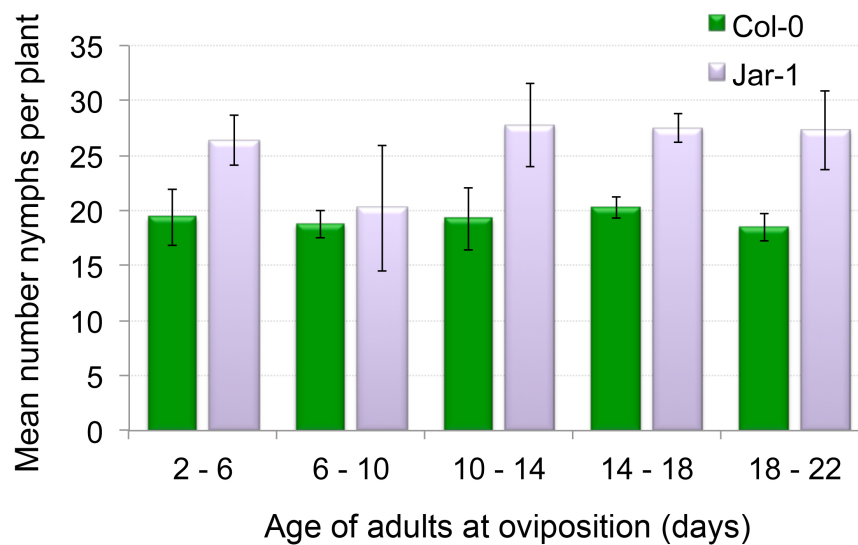


**Figure. 4.2.7.2. *M. quadrilineatus* feed/probe sites numbers are unchanged by reduction of LOX2**

Feed/probe sites were examined as visual measure of insect feeding of Arabidopsis to assess whether leafhoppers have differential feeding on Arabidopsis with silenced LOX2 expression. Five *M. quadrilineatus* were enclosed on LOX2 and Col-0 single leaves for 5 days. Leaves were then cut and stained with trypan to render feed/probe sites visible in blue. Total feed/probe sites were counted on each leaf. More feed/probe sites were found on LOX2 silenced Arabidopsis, however this increase was not significantly different from numbers on Col-0 controls  $F_{1,33} = 2.654$ ,  $P = 0.394$ . This suggests that *M. quadrilineatus* feeding is not affected by silenced LOX2. Modeled by plant line using GLM with a poisson link function. Bars = 2x standard error above and below means.

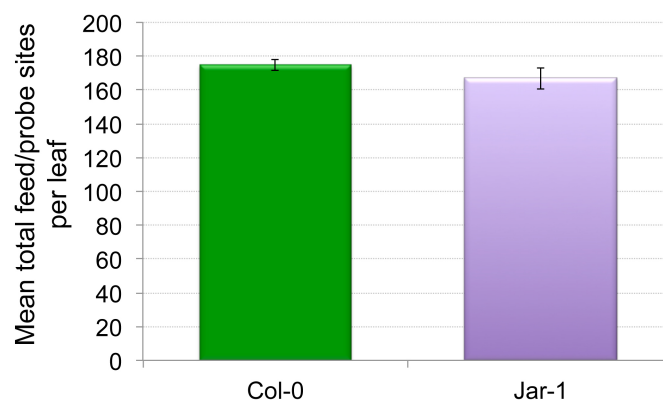
Leaf Treatment	Mean total feed/probe sites per leaf			
	Primary vein	Secondary vein	Tertiary veins	Open Tissue
Col-0	96.50	37.88	26.00	17.38
	+/- 3.12	+/- 3.27	+/- 2.43	+/- 2.55
LOX2	97.63	38.20	30.38	15.35
	+/- 2.83	+/- 3.76	+/- 1.477	+/- 3.41

**Table 4.2.7.3. *M. quadrilineatus* feed/probe sites are in similar locations on LOX2 and Col-0 leaves.** Leaves were exposed to leafhoppers and stained with trypan blue to highlight feed/probe sites were examined under a Nikon E800 fluorescent microscope. Feed/probe sites were counted on primary, secondary, tertiary veins, and open tissue of leaves. *M. quadrilineatus* concentrated feeding on the primary veins  $F_{4,16} = 3.743$ ,  $P < 0.001$ . No significant difference was observed in feed/probe site locations between LOX2 and Col-0 Arabidopsis  $F_{1,19} = 37.401$ ,  $P = 0.836$ . Data were modeled by plant line using GLM with a poisson link function for count data. Errors are 2x standard error above and below the predicted means.



**Figure. 4.2.7.4. *M. quadrilineatus* fecundity is increased on Arabidopsis with compromised JA synthesis.**

Assays were carried out on Arabidopsis with reduced Jasmonic acid production to assess whether the compromise of down stream targets of SAP11, also has an effect on *M. quadrilineatus* fecundity. Eight female and 2 male *M. quadrilineatus* were added to each experimental plant from 4 days. Numbers of nymphs were counted 15 days after adults were removed from plants to allow them to develop to at least 2<sup>nd</sup> instar. The experiment included 3 plants of each plant line per 5 leafhopper age ranges and the whole experiment was repeated 3 times (90 plants and 900 leafhoppers). *M. quadrilineatus* fecundity was significantly increased on Jar1 Arabidopsis compared with Col-0  $F_{1, 71} = 8.0376$ ,  $P = <0.001$ . Age of leafhoppers at oviposition also had a significant increasing effect  $F_{4, 71} = 15.308$ ,  $P = <0.001$ . Modeled by rep\*plant line\*age using GLM with a poisson link function. Bars = 2x standard error above and below means.



**Figure 4.2.7.5. *M. quadrilineatus* feed/probe less on Arabidopsis with reduced JA synthesis.**

Feed/probe sites were examined as visual representation of insect feeding of Arabidopsis to assess any differential feeding on Arabidopsis with reduced JA synthesis. Five *M. quadrilineatus* were enclosed on LOX2 and Col-0 single leaves for 5 days. Leaves were then cut and stained with trypan to render feed/probe sites visible in blue. Total feed/probe sites were counted on each leaf. Significantly less feed/probe sites were found on Jar-1 Arabidopsis compared with Col-0  $F_{1,27} = 15.378$ ,  $P = 0.01$ . This suggests that *M. quadrilineatus* may be feeding less on Arabidopsis with reduced JA synthesis. Reduced feeding could indicate that the leafhoppers feed more easily or efficiently on Jar-1. Modeled by plant line using GLM with a poisson link function. Bars = 2x standard error above and below means.

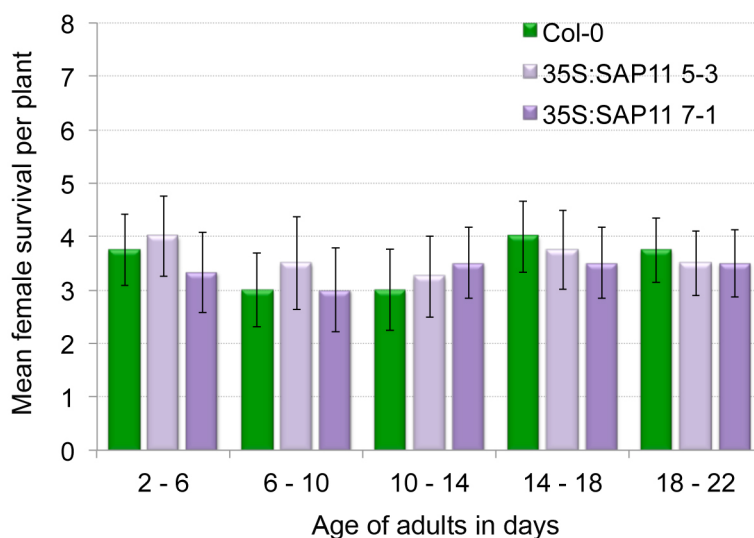
Leaf Treatment	Mean total feed/probe sites per leaf			
	Primary vein	Secondary vein	Tertiary veins	Open Tissue
Col-0	116.13	25.13	17.13	16.25
	+/- 2.56	+/- 2.013	+/- 1.97	+/- 1.732
Jar1	116.00	24.75	15.13	11.00
	+/- 3.11	+/- 1.24	+/- 2.16	+/- 1.524

**Table 4.2.7.6. *M. quadrilineatus* feed/probe in similar locations on Jar-1**

**Arabidopsis.** Leaves were exposed to leafhoppers and stained with trypan blue to highlight feed/probe sites were examined under a Nikon E800 fluorescent microscope. Feed/probe sites were counted on primary, secondary, tertiary veins, and open tissue of leaves. *M. quadrilineatus* concentrated feeding on the primary veins  $F_{4,24} = 5.786$ ,  $P < 0.001$ . No significant difference was found in the locations of feed/probe on Col-0 controls and Jar-1 Arabidopsis  $F_{1,22} = 7.687$ ,  $P = 0.763$ . This indicates that feed site location behaviour is unaffected by reduction in JA. Modeled by plant line using GLM with a poisson link function. Errors shown are 2x standard error above and below means.

#### 4.2.8 AY-WB secreted proteins play different roles in host and non-host interactions

Host leafhopper *M. quadrilineatus* has increased fecundity on Arabidopsis with the AY-WB secreted protein *SAP11* stably expressed (Sugio *et al.* 2011). Furthermore, *M. quadrilineatus* has increased fecundity on Arabidopsis in-which downstream targets of *SAP11*, (*LOX2* and ultimately the plant defence hormone jasmonic acid) are compromised (Sugio *et al.* 2011). Here *D. maidis* was examined to assess whether survival and fecundity of these non-host leafhoppers could also be increased by expression of *SAP11* or the compromise of *LOX2* or *JA* in non-host Arabidopsis. Expression of *SAP11* made no significant difference to the survival of *D. maidis* (Fig. 4.2.19) and no eggs or nymphs were recorded on 35S:*SAP11* Arabidopsis. The compromise of *LOX2*, although shown to increase attraction of novel herbivores to *Nicotiana attenuata* (Kessler *et al.* 2004), had no effect on the survival of *D. maidis* (Fig. 4.2.20) and no eggs or nymphs were recorded on plants. Interestingly, *D. maidis* survival was increased on *Jar-1* Arabidopsis with compromised production of jasmonic acid (Fig. 4.2.8.1), although, no eggs or nymphs were recorded.

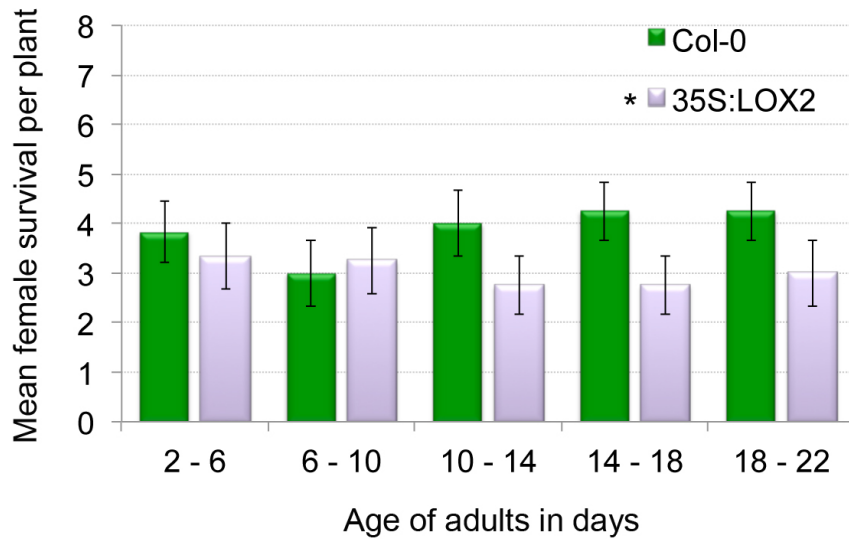


**Figure 4.2.8.1. AY-WB secreted protein *SAP11* has no effect on *D. maidis* survival**

Eight females and 2 males were added to 7 week old experimental plants for 4 days, after which time surviving females were counted. Three plants per plant line were

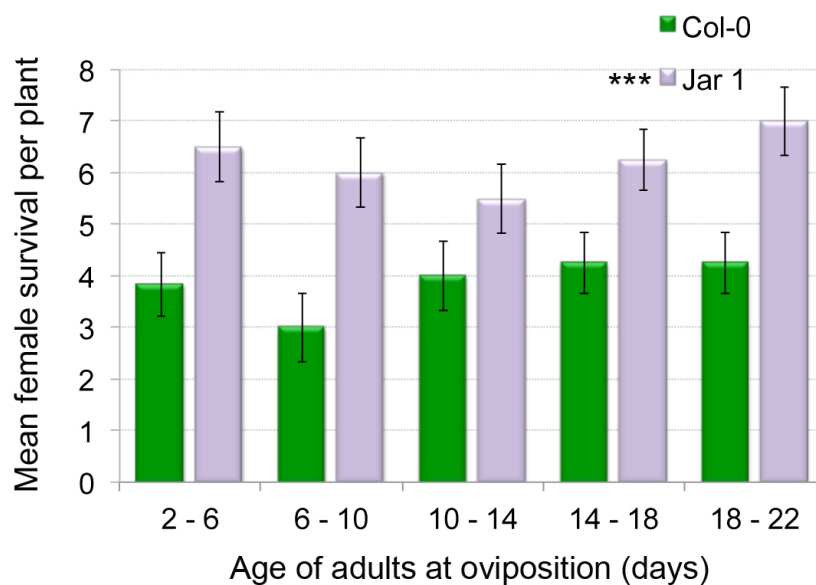


examined for each leafhopper age group and the whole experiment was repeated 3 times. *D. maidis* survival was not significantly altered on 35S:SAP11 lines compared to Col-0 Arabidopsis  $F_{2,45} = 0.4206$   $P = 0.153$ . Age of the insects also had no significant effect on survival on these lines  $F_{4,45} = 0.0950$   $P = 0.778$ . No nymphs were produced on any of these lines so fecundity was not affected. Modeled by rep\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 4.2.8.2. Compromised up-regulation of LOX2 in Arabidopsis has a negative effect on *D. maidis* survival.**

Eight females and 2 males were added to 7 week old experimental plants for 4 days, after which time surviving females were counted. Three plants per plant line were examined for each leafhopper age group and the whole experiment was repeated 3 times. Survival of *D. maidis* Arabidopsis with compromised LOX2 (35S:LOX2) was marginally significantly less than survival on Col-0 controls,  $F_{1,24} = 3.7844$   $P = 0.052$ . Age of the insects did not significantly affect survival  $F_{4,24} = 0.1758$   $P = 0.951$ . No nymphs were produced on any of these lines so fecundity was not affected. These data suggest that silenced LOX2 has a negative effect on the non-host interaction between *D. maidis* and Arabidopsis. Modeled by rep\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 4.2.8.3. *D. maidis* has increased survival on *Arabidopsis* with compromised production of defence hormone jasmonic acid**

*D. maidis* survival on *Arabidopsis* with compromised jasmonic acid production (Jar-1) was increased compared with Col-0,  $F_{1,24} = 35.5318$   $P < 0.001$ . Age had no effect on survival on these lines  $F_{4,24} = 0.8435$   $P = 0.497$ . No nymphs were produced on either of these lines so fecundity was not affected. Modeled by  $\text{rep} \times \text{plant line} \times \text{age}$  using GLM with a poisson link function. Errors shown are 2x standard error above and below means.

## Discussion

### 4.3.1 SAP11 within *Arabidopsis* manipulates vector fitness

The AY-WB secreted effector protein SAP11 destabilises *Arabidopsis* TCPs and downstream synthesis of LOX2 and jasmonic acid (Sugio *et al.* 2011). *M. quadrilineatus* has increased fecundity on *Arabidopsis* with stable expression of 35S:SAP11. Therefore, AY-WB secreted SAP11 plays a part in rendering *Arabidopsis* more susceptible to herbivory or oviposition of *M. quadrilineatus*. However, this increase is not as high as the increase recorded on AY-WB infected *Arabidopsis*. Furthermore, SAP11 stable expression using the 35S promoter supports SAP11 expression throughout the plant at high levels. This level of protein abundance is not realistic to AY-WB infection, but it does yield an important clue as to SAP11 function in the plant, and in modulation of host plant interactions. It is likely that SAP11 plays a part role in the increase in vector fecundity on AY-WB infected plants.

Feeding on 35S:SAP11 plants is not significantly altered compared with uninfected. This suggests that SAP11 may not affect *M. quadrilineatus* feeding but may affect suitability of the plant for oviposition. *M. quadrilineatus* also has increased fecundity on LOX2 deficient and TCP deficient *Arabidopsis*. Thus, gives confirmation of the mode of action and contribution of SAP11 in AY-WB infected *Arabidopsis*.

Inducible 35S:SAP11 using the Cre-Lox system required treating plants with heat shock and dex, both of which contributed to increased *M. quadrilineatus* fecundity, thus masking any effect of inducible SAP11. Leafhopper fecundity was unaffected or negatively affected on *Arabidopsis* with stable expression of 35S:SAP11 with altered nuclear localization or nuclear export signals (35S:SAP11  $\Delta$ NLS3 NES and 35S:SAP11 NESKO). Indicating that SAP11 requires on nuclear localization and nuclear export signals for proper function. AY-WB uses the secreted effector protein SAP11 to modulate host plant defence via destabilization of TCPs and jasmonic acid synthesis. Thus, SAP11 plays

a clear part in manipulation of insect-plant interactions stemming from the plant.

#### *4.3.2 Warmer climate conditions mask SAP11 efficiency*

The migratory leafhopper *M. quadrilineatus* encounters warm conditions in the field that can affect insect fitness including fecundity and development (Hoy *et al.* 2003; Beanland *et al.* 2005). To assess the affect of SAP11 in warmer conditions, fecundity experiments were repeated in 3 different environmental conditions. *M. quadrilineatus* exhibited increased fecundity on all plants in warmer conditions. Therefore masking the effect of SAP11 and blurring whether the effect originated from heat stressed plants or heat induced oviposition in the insects. AYPs transmission can be compromised at prolonged high temperatures, even as the vector fitness increases (Goodwin *et al.* 1999). Further examination of SAP11 function in higher temperatures may be worthwhile, as it is feasible that SAP11 function is compromised in high temperatures. Aster yellows phytoplasmas in Canada have compromised transmission in hotter than usual season conditions indicating that high temperature may interfere with phytoplasma transmission or replication in hosts (Goodwin *et al.* 1998). It is possible that protein secretion or function may be compromised in these conditions.

#### *4.3.4 SAP11 plays different roles in host and non-host interactions*

Host leafhopper *M. quadrilineatus* has increased fecundity on Arabidopsis with the AY-WB secreted protein *SAP11* stably expressed (Sugio *et al.* 2011). Furthermore, *M. quadrilineatus* has increased fecundity on Arabidopsis in-which downstream targets of *SAP11*, (LOX2 and ultimately the plant defence hormone jasmonic acid) are compromised (Sugio *et al.* 2011). *D. maidis* survival and fecundity was assessed to see whether it is affected by expression of *SAP11* or the compromise of LOX2 or JA in non-host Arabidopsis (LOX2 silenced or Jasmonate resistant 1 [Jar-1]. *SAP11* expression had no impact on the

survival of *D. maidis* and no eggs or nymphs were recorded on any experimental plants. The compromise of LOX2, although shown to increase attraction of novel herbivores to *Nicotiana attenuata* (Kessler *et al.* 2004), had no effect on the survival of *D. maidis* and no eggs or nymphs were recorded. Interestingly, *D. maidis* survival was increased on Jar-1 Arabidopsis with compromised production of jasmonic acid, although no eggs or nymphs were recorded. Whilst SAP11 destabilizes TCPs and jasmonic acid production, reducing plant defence to *M. quadrilineatus*, *D. maidis* is unaffected by SAP11 manipulations of non-host Arabidopsis. This indicates that other SAPs may play a part in enabling *D. maidis* to survive and reproduce on non-host Arabidopsis.

#### 4.3.5 Summary

*M. quadrilineatus* has significantly higher fecundity on Arabidopsis with stably expressed SAP11 than Col-0 controls. This suggests that SAP11 plays a role in increasing *M. quadrilineatus* fecundity on AY-WB infected Arabidopsis. However, in 35S::SAP11 Arabidopsis, SAP11 is found throughout the plant in relatively higher concentration than levels in AY-WB infected Arabidopsis. Thus, examining fecundity on Arabidopsis with expression of SAP11 using a phloem-limited promoter such as the AtSUC2 promoter, may give a more realistic indication of the effect that SAP11 has on leafhopper plant interactions.

Whilst SAP11 has an effect on *M. quadrilineatus* vector fecundity on plants in 22°C with 14 hour day, this effect is lost in warmer conditions. SAP11 efficacy may be temperature dependant or this may be an indication that SAP11 plays a part role in AY-WB modulation of host plants, thus need other AY-WB secreted proteins to affect TCPs and downstream targets such as LOX2 and JA.

SAP11 is encoded on a pathogenicity island with 5 other proteins and these may interact or at least have an additive effect on modulation of plant hosts. Further experiments on using expression of SAP11 together with other SAPs on this island could yield further understanding of the function of this group of secreted proteins within plant hosts.

*D. maidis* is unaffected by expression of SAP11 in Arabidopsis, but has higher survival on AY-WB infected Arabidopsis. This supports the hypothesis that SAP11 function is part of a larger complex of AY-WB secreted proteins that affect insect-plant interactions by modulating processes within the host plant. Further study of SAPs on the same pathogenicity island as SAP11, may yield clues as to the roles of these proteins in increasing non-host herbivore interactions.

## **Chapter five: AY-WB secreted proteins play different roles in host and non-host manipulation.**

### **Introduction**

#### *5.1.1 AY-WB SAP11 manipulates plant defence and morphology to alter vector fitness*

The previously sequenced AY-WB genome was mined and 56 candidate effectors were identified (Bai *et al.* 2008). The AY-WB effector SAP11 modulates plant defence responses to the advantage of the AY-WB insect vector *M. quadrilineatus*. SAP11 binds and destabilizes Arabidopsis *CINCINNATA* (*CIN*)-related TCP transcription factors, which control plant development and promote the expression of lipoxygenase (*LOX*) genes involved in jasmonate (JA) synthesis. Both the Arabidopsis SAP11 lines and AY-WB-infected plants produce less JA upon wounding. Furthermore, the AY-WB insect vector produces more offspring on AY-WB-infected plants, SAP11 transgenic lines and plants impaired in *CIN*-TCP and JA synthesis. Thus, SAP11-mediated destabilization of *CIN*-TCPs leads to the downregulation of *LOX2* expression and JA synthesis and an increase in *M. quadrilineatus* progeny. Phytoplasmas are obligate inhabitants of their plant host and insect vectors in which the latter transmits AY-WB to a diverse range of plant species. This finding demonstrates that pathogen effectors can reach beyond the pathogen-host interface to modulate a third organism in the biological interaction.

#### *5.1.2 All 56 AY-WB effector proteins may contribute to host manipulation*

Bai *et al.* identified 56 secreted proteins and 20 membrane proteins that may play roles in plant or insect host manipulation (Bai *et al.* 2008). MacLean *et al.* reported that 20 of these secreted proteins are secreted more in AY-WB infected Arabidopsis and 16 are secreted more in AY-WB infected *M. quadrilineatus* (Fig. 6.1.2.1) (MacLean *et al.* 2011). The knowledge of which proteins are secreted in insect or plant hosts

yields a reasonable assumption as to the general target of the secreted proteins. Proteins secreted with in infected plants may all play roles in plant host manipulation. Expressing the genes encoding these proteins in *Arabidopsis* allows their function to be examined. Maclean *et al.* demonstrate that expression of SAP54 (AYWB\_224), secreted predominantly into AY-WB infected plants, results in altered flower morphology. They show that *Arabidopsis* carrying the 35S::SAP54 transgene repeatedly form flowers within flowers (a secondary flower is generated in the forth whorl in lieu of a carpel (Fig. 5.1.2.2A). This phenotype is deemed to be associated with the loss of determinacy of the flower meristem. In addition 35S::SAP54 *Arabidopsis* flower petals were generally virescent (Fig. 5.1.2.2B), and sepals were leaf-like and covered in trichomes (Fig. 5.1.2.2C) (MacLean *et al.* 2011). This change in flower morphology is similar to that exhibited by AY-WB infected *Arabidopsis* (Fig. 5.1.2.3). The similarity in altered flowers suggests that SAP54 plays an important role in manipulating flower development.

AY-WB Gene <sup>a</sup>	ID <sup>b</sup>	Fold increase in insect <sup>c, d</sup>	Fold increase in plant <sup>c, d</sup>	P-value <sup>d</sup>
pl03	SAP37		1.3	0.148
22	SAP21		1.7	0.209
32	SAP05		2.2	0.007
33	SAP06		1.8	0.247
73	SAP19	2.6		0.005
127	SAP27		29.4	0.01
145	SAP59		1.8	0.094
146	SAP26		2.1	0.01
148	SAP25	1.0		0.869
169	SAP61		12.3	0.007
189	SAP36	21.1		0.006
203	SAP55		1.4	0.093
212	SAP39	1.5		0.25
224	SAP54 *		1.4	0.028
225 <sup>e</sup>	SAP53	1.9		0.01
229 <sup>e</sup>	SAP62	1.7		0.008



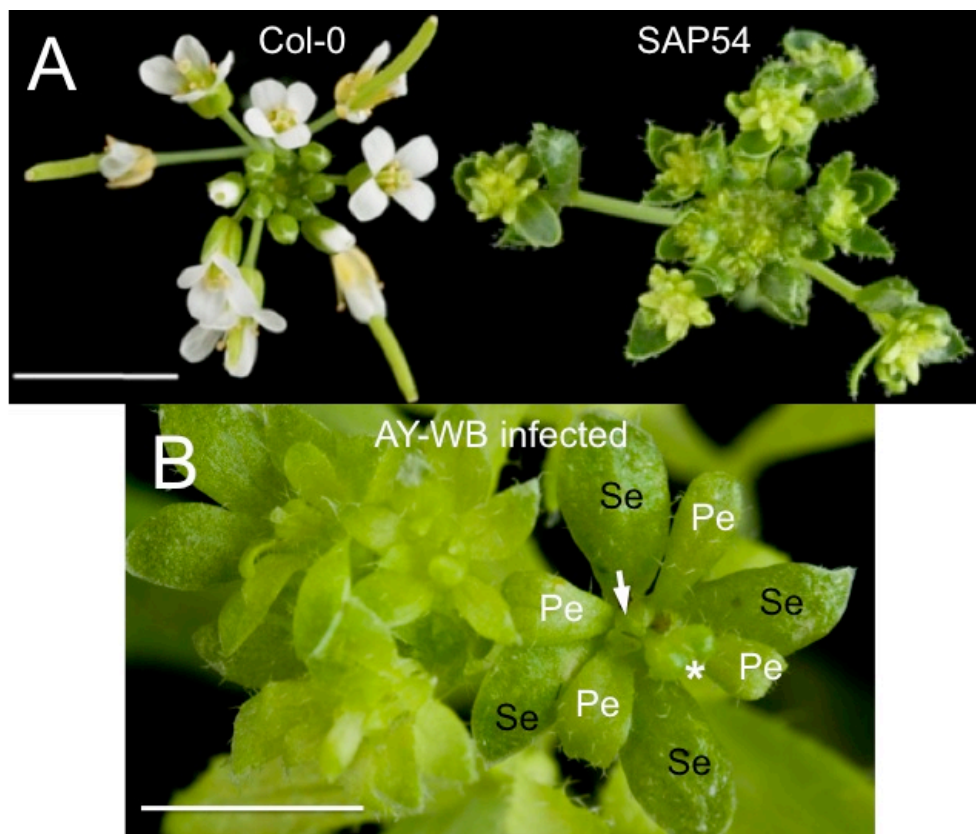
236	SAP40		4.1	0.007
237	SAP41		6.5	0.013
245	SAP63	2.9		0.003
259	SAP43		3.2	0.014
263	SAP52	1.6		0.003
269	SAP64	11.8		0.002
275	SAP22		1.0	0.833
280	SAP44		1.4	0.099
294	SAP45		1.4	0.23
295	SAP51	3.7		0.003
329	SAP65	7.3		0.003
339	SAP49		2.1	0.004
340	SAP48		1.5	0.081
342 <sup>e</sup>	SAP47		3.3	0.007
366	SAP66 *		2.1	0.009
367 <sup>e</sup>	SAP56 *		2.4	0.01
368 <sup>e</sup>	SAP67 *		2.7	0.01
369	SAP68 *		2.6	0.014
370	SAP11 *		2.5	0.01
376 <sup>e</sup>	SAP09 *	1.8		0.082
387	SAP08	8.8		0.003
402	SAP30		3.3	0.011
433	SAP50	5.6		0.005
480	SAP69	1.8		0.012
529	SAP70	1.5		0.003
562	SAP34	2.3		0.003
568	SAP71		1.3	0.002
588	SAP35		1.3	0.014
624	SAP15	1.9		0.008
640	SAP13		2.5	0.011
645	SAP20		10.9	0.013
667	SAP72	1.2		0.003

**Figure. 5.1.2.1. AY-WB secreted proteins are differentially secreted in insect and plant hosts**

Figure adapted from MacLean *et al.* 2011, experiment completed by O. V. Makarova, Department of Agroecology, Faculty of Science and Technology, Aarhus University, Slagelse, DK-4200, Denmark.

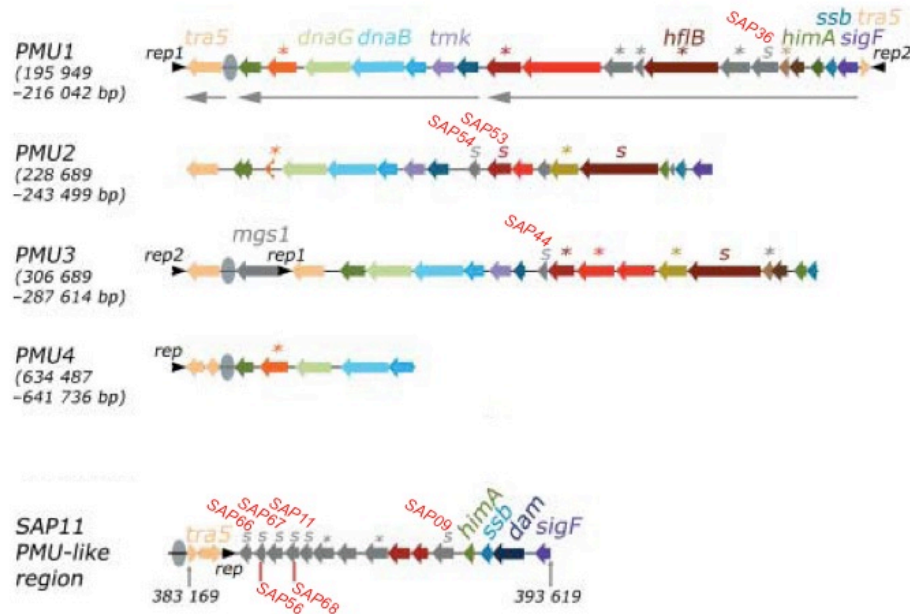
\* Secreted proteins discussed in this chapter. <sup>a</sup> Numbers refer to locus tags of GenBank accessions NC\_0007716 (AYWB genes on chromosome), and NC\_007717 (pI03 on AY-WB plasmid).

<sup>b</sup> Identities (ID) correspond to secreted AY-WB proteins (SAPs), which are candidate effectors, published in Bai et al., 2009 (Tables 1 and 2). <sup>c</sup> The expression levels of the AY-WB genes shown in this table were normalized with those of *AYWB\_007* (*dnaB*), *AYWB\_064* (*ftsY*), *AYWB\_254* (*gyrA*), *AYWB\_434* (*pykF*) and *AYWB\_440* (*pfkA*). <sup>d</sup> Table rows of genes up regulated in plants versus insects are highlighted in green and those up-regulated in insects versus plants in light yellow (p-value < 0.05). Genes whose expression is not significantly different between the plant and insect host are represented in white rows. <sup>e</sup> Due to identical sequences in the primer annealing sites, primers may not be gene specific and may have amplified more than one target.



**Figure. 5.1.2.2. SAP54 plays a role in AY-WB manipulation of host floral organs**  
**A**, Images of inflorescences of Col-0 (left) and 35S::SAP54 line 4 transgenic Arabidopsis (middle and right). Transgenic plants grown in cool conditions at 22°C consistently with a 10 hour light and 14 hour dark (right) show indeterminate flower growth (flowers within flowers). Scale bar; 0.5 cm. Figure **A** taken from MacLean *et al.* 2011, experiment completed by A. MacLean at the John Innes Centre, Norwich Research Park, England. **B** demonstrates AY-WB Arabidopsis with altered flower morphology. **B** plants grown in cool conditions 22°C 10hr L/14 hr D and reflect typical flower morphology of AY-WB infected Arabidopsis. Enlarged sepals (Se), Green petals with trichomes (Pe), reduced stamens (white arrow), and in many flowers fused stamen or fused stamen and carpels (asterisk). Scale bar; 0.2cm. Figure **B** reinforces

findings of MacLean *et al* that AY-WB manipulation of floral organs is similar to SAP54 manipulation.



**Figure. 5.1.2.3. AY-WB potential mobile units hold genes for secreted proteins**

AY-WB secreted proteins highlighted in red on potential mobile units (PMUs) of the AY-WB chromosome and the PMU-like region encoding SAP11. The chromosome is presented as a black line. ORFs are represented as block arrows in which paralogous genes have the same colours, with the exception of the grey-coloured arrows that represent unique genes. Conserved 395-bp regions upstream of *tra5* genes are indicated as grey vertical transparent ovals. The locations and directions of the ~330-bp conserved repeats (rep) are indicated in closed black arrowheads. The names of the ORFs with predicted functions are indicated above the arrows, with ORFs of predicted membrane-targeted proteins indicated with an asterisk and predicted secreted proteins with 's'. The *tra5* ORFs of PMU4 and the SAP11 PMU-like region contain separate A and B ORFs that may produce a full-length transposases upon single frameshifting events. The three transcripts predicted for PMU1 are indicated as thin grey arrows beneath the PMU1 region. Figure modified from Hogenhout *et al*, 2008.

### 5.1.3. Appraisal of roles of 5 effector proteins encoded on the SAP11 PMU-like region

Bai *et al.* sequenced the genome of AY-WB and described 16 potentially secreted or membrane targeted proteins encoded on 4 repeated regions described as potential mobile units (PMUs) of the chromosome (Bai *et al.* 2006). In 2008, Bai *et al.* identified 56 genes for candidate secreted AY-WB proteins (SAPs), 4 of which are encoded on the PMUs (Fig. 5.1.2.3), 7 are encoded on plasmids, 6 on an additional PMU-like region and 45 elsewhere on the chromosome (Bai *et al.* 2006; 2009). SAP36 on PMU1 is expressed highly in *M. quadrilineatus* and potentially plays a role in insect host manipulation (Maclean *et al.* 2011). SAP53 and SAP54 on PMU2 are expressed highly in *M. quadrilineatus* and Arabidopsis respectively and SAP54 clearly plays a role in flower manipulation (Fig. 5.1.2.2) (MacLean *et al.* 2011). SAP44 on PMU3 is expressed at a similar level in insects and plants and its function is unknown (MacLean *et al.* 2011). SAP05 is not found on a PMU or plasmid but is expressed highly in plants and generates a heart shaped leaf phenotype when expressed in Arabidopsis (Sugio, unpublished data). SAP11 on the SAP11 PMU-like region is expressed highly in Arabidopsis, targets plant cell nuclei, and affects plant morphology and defence leading to increased leafhopper oviposition (Bai *et al.* 2009; Sugio *et al.* 2011). The 6 SAPs on the SAP11 PMU-like region are all significantly expressed in plant hosts (with the exception of SAP09 which is found in insect and plant hosts) and are transcribed with SAP11 (Bai *et al.* 2009). This indicates that the other SAPs on this PMU may also play a role in host manipulation although when individually expressed in Arabidopsis no lines show changes in plant morphology (Sugio, unpublished data). SAP56, 66, 67, 68, 11 and membrane protein AYWB371 are translated from one transcript and transcribed simultaneously in plants (Bai *et al.* 2009). Thus, proteins encoded on this transcript probably perform their roles at the same point of infection within the plant and may even work together. In this chapter I

demonstrate that SAPs on the SAP11 PMU-like region and SAP05 play differential roles in leafhopper-Arabidopsis interactions.

## Results

### 5.2.1 SAP56 renders *Arabidopsis* more susceptible to *D. maidis*

AY-WB secreted protein SAP11 plays a clear role in phytoplasma manipulation of insect-plant interactions. To assess whether the other secreted proteins encoded on the SAP11 PMU-like region also play roles in host interaction manipulation, survival and fecundity trials were conducted on transformed *Arabidopsis*. Stable expression lines of *Arabidopsis* Col-0 35S::SAP56, 35S::SAP66, 35S::SAP67, 35S::SAP68 (all translated together with SAP11), 35S::SAP09 (from the SAP11 PMU-like region) and 35S::SAP05 (which generates a heart shaped leaf phenotype in *Arabidopsis*). In survival and fecundity trials control plants were 35S::SAP11 lines and Col-0. In all experiments, *M. quadrilineatus* survival was high and not significantly different from previous experiments.

In these experiments leafhopper fecundity was noticeably more variable than recorded previously, on control plants and on newly tested *Arabidopsis* lines (Fig. 5.3.1.1, 5.2.2.1). Some plants were yielding over 70 nymphs. I chose to add a number of controls that may highlight contributory factors to variation in fecundity data. Experiments were repeated to include warmer temperatures, addition and omission of CO<sub>2</sub> anesthesia of leafhoppers (to sex), and the use of two different host plants for rearing insects prior to experiments.

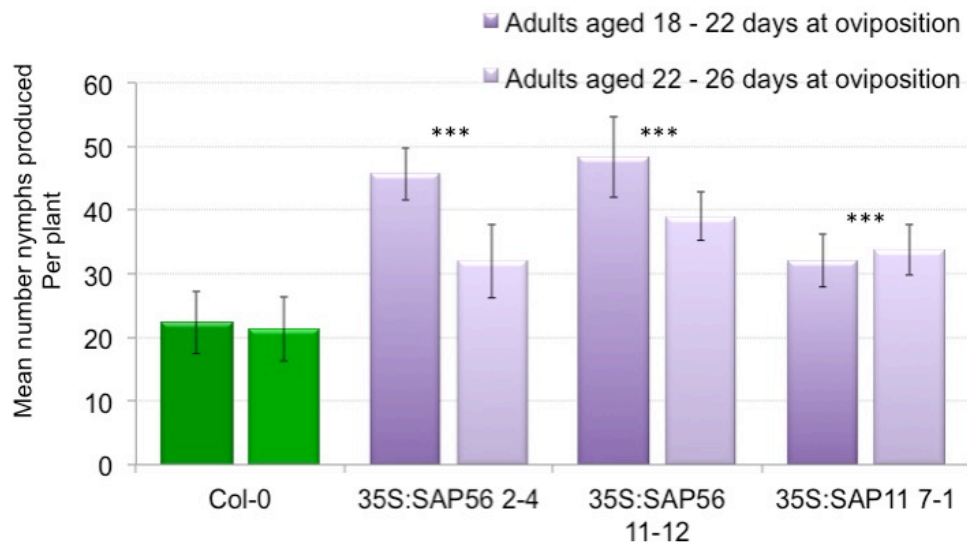
Temperature and day length have been shown to alter *M. quadrilineatus* reproduction, movement and dispersal (Beanland *et al.* 2005; Larson *et al.* 1990). Use of CO<sub>2</sub> anesthesia to sex leafhoppers may also affect *M. quadrilineatus* as it is reported to affect insects short-term metabolism, fecundity and ability to vector viruses (MacAlpine *et al.* 2011; Mbata *et al.* 1998). Environmental changes are known to affect metabolism, defence gene regulation, oviposition and survival of insects (Schmid-

Hempel 2004). Therefore, addition of these variables within the experiment more robustly tests the impact of proteins on leafhopper plant interactions.

In initial assays, SAP56 had a significant increasing effect on *M. quadrilineatus* fecundity (Fig. 5.3.1.2). A few 35S::SAP56 plants produced over 50 nymphs, as such, these multiple outliers were not excluded from data and therefore had an impact on results and variability. Fecundity on 35S::SAP11 Arabidopsis was more variable in these experiments but remained significantly higher compared with Col-0.

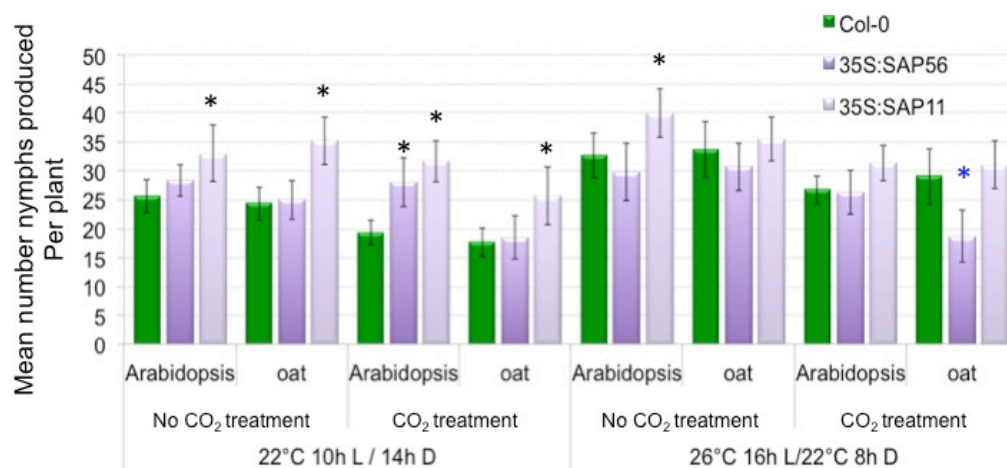
In the second set of experiments I found that CO<sub>2</sub> significantly affected leafhopper fecundity in cooler conditions on control Col-0 Arabidopsis (Fig. 5.3.1.2). Furthermore, *M. quadrilineatus* fecundity was significantly reduced by CO<sub>2</sub> treatment on 35S::SAP56. However, leafhoppers raised on Arabidopsis (had no host change at the start of the experiment) this effect was masked. Thus, a host change and CO<sub>2</sub> anesthesia has an additive negative effect on *M. quadrilineatus*. Interestingly, *M. quadrilineatus* have significantly higher fecundity in warmer longer day conditions (Fig. 5.3.1.2) confirming results in earlier chapters. The increase in fecundity on all Arabidopsis lines masks the increase in fecundity seen on 35S::SAP11 (Fig. 5.3.1.2). SAP56 is only effective in increasing *M. quadrilineatus* fecundity when leafhoppers are challenged with CO<sub>2</sub> and no host change. It is plausible that SAP56 has a more biologically significant effect when expressed together with other proteins on the SAP11 pathogenicity island.

Interestingly, SAP56 does play a role in rendering Arabidopsis more susceptible to maize specialist leafhopper *D. maidis* (Fig. 5.3.1.3). *D. maidis* survival is increased on 35S::SAP56 Arabidopsis,  $F_{1,31} = 6.902$   $P = 0.001$ . *D. maidis* did oviposit only 3 eggs on 2 35S::SAP56 plants but no eggs hatched so fecundity is unaffected by SAP56. These results indicate that SAP56 may play a greater role manipulating plant defence against non-host insects.



**Figure 5.3.1.1. SAP56 has a significant effect on *M. quadrilineatus* fecundity**

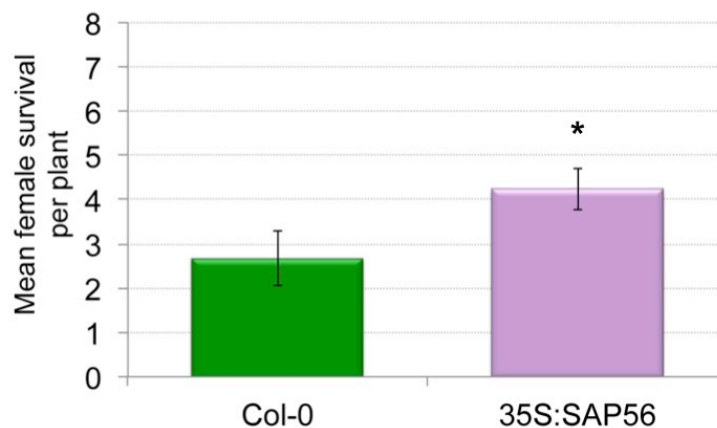
*M. quadrilineatus* fecundity was assessed on 35S:SAP56 Arabidopsis and Col-0 and 35S:SAP11 as controls. Nymphs production was significantly higher 335S:SAP56 and Col-0,  $F_{2,43} = 64.362$   $P = 0.001$ . Error bars highlight the variability of the data and that a few plants had over 50 nymphs, however many plants produced similar yield of nymphs as those on control Col-0 plants. SAP11 lines yielded higher nymph numbers than Col-0 as in previous experiments,  $F_{1,67} = 5.3108$   $P = <0.001$ . Modeled by rep\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.3.1.2. SAP56 has a significant effect on *M. quadrilineatus* fecundity only when insects are challenged with CO<sub>2</sub> and cool conditions.**

In these assays, 2 temperature contitions were included 22°C 10h day/14h night and 26°C h day/8h night. Four treatments were included, leafhoppers reared on Oats and

treated with CO<sub>2</sub> and leafhoppers reared on Oats not treated with CO<sub>2</sub>, leafhoppers reared on Arabidopsis and treated with CO<sub>2</sub> and finally leafhoppers reared on Arabidopsis and not treated with CO<sub>2</sub>. *M. quadrilineatus* fecundity was tested on 2 35S:SAP56 lines with these treatments and condition together with Col-0 and 35S::SAP11 as control plants. Leafhoppers in experiments were 14-18 days old and 18-22 days old (means of plant lines and ages are shown). Insect age had no significant effect on *M. quadrilineatus* fecundity  $F_{1,97} = 21.7058$   $P = 0.387$ . Fecundity was only significantly increased on 35S:SAP56 Arabidopsis when leafhoppers were reared on Arabidopsis (so had no host change) anesthetized with CO<sub>2</sub> and kept in cool conditions (22°C 10h L/ 14h D)  $F_{4,47} = 10.583$   $P = 0.027$ . This suggests that SAP56 has no biologically significant effect on *M. quadrilineatus* fecundity. SAP11 lines yielded higher nymph numbers than Col-0 in cool conditions, as in previous experiments,  $F_{1,57} = 15.732$   $P = 0.01$ . Modeled by rep\*treatment\*condition\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.1.3. SAP56 increases *D. maidis* survival on Arabidopsis**

*D. maidis* survival was measured on Arabidopsis 35S:SAP56 and Col-0 controls, survival was increased on lines;  $F_{1,36} = 4.7785$   $P = 0.001$ , respectively. A total of 3 eggs were found only on two 35S:SAP56 plants, however none hatched. Therefore no significant increase in fecundity was observed on these lines. Modeled by rep\*plant line\*age using GLM with a binomial link function. Errors shown are 2x standard error above and below means.

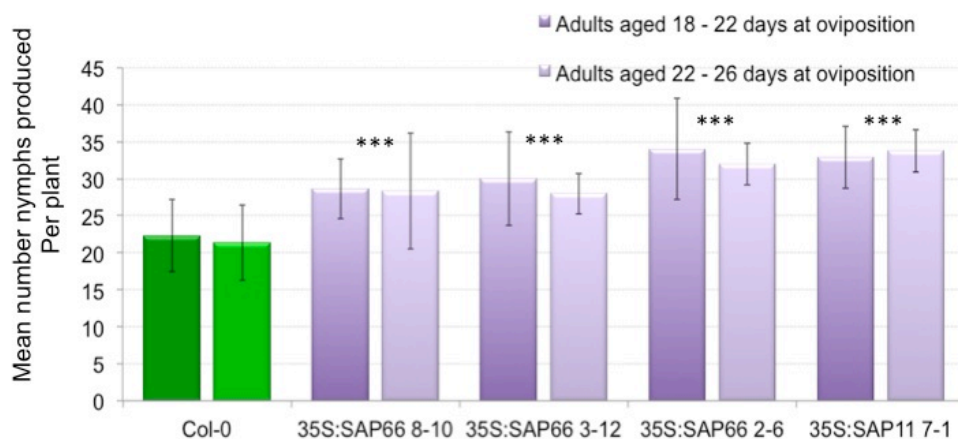
### 5.2.2 *D. maidis* has increased survival on SAP66 Arabidopsis

SAP66 had a significant increasing effect on *M. quadrilineatus* fecundity (Fig. 5.2.2.1). Over 40 nymphs were produced on occasional 35S::SAP66



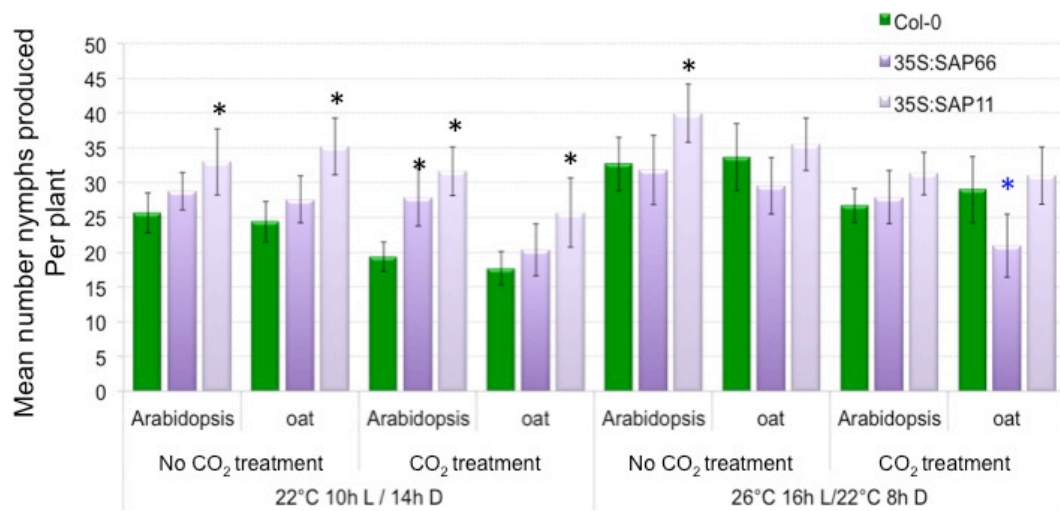
these are highlighted by error bars (Fig. 5.2.2.1). These outliers made a significant impact on results. In addition, I found CO<sub>2</sub> significantly affected leafhopper fecundity in cooler conditions on control Col-0 Arabidopsis (Fig. 5.2.2.2). Furthermore, *M. quadrilineatus* fecundity was significantly reduced by CO<sub>2</sub> treatment on 35S::SAP66, However, for leafhoppers raised on Arabidopsis (no host change at the start of the experiment) this effect was masked,. Thus, a host change and CO<sub>2</sub> anesthesia has an additive negative effect on *M. quadrilineatus*. Interestingly, *M. quadrilineatus* have significantly higher fecundity in warmer longer day conditions (Fig. 5.2.2.2) confirming results in earlier chapters. The increase in fecundity on all Arabidopsis lines masks the increase in fecundity seen on 35S::SAP11 (Fig. 5.2.2.2). SAP66, like SAP56 appears to only be effective in increasing *M. quadrilineatus* fecundity when leafhoppers are challenged with CO<sub>2</sub> and no host change. This suggests that SAP66 has an insignificant effect on vector fecundity but does not rule out its' potential role in co-expression with other proteins.

SAP66 does play a role in rendering Arabidopsis more susceptible to maize specialist leafhopper *D. maidis* (Fig. 5.2.2.3). *D. maidis* survival is increased on 35S::SAP66 Arabidopsis,  $F_{1,36} = 3.748$   $P = 0.005$ . No eggs or *D. maidis* nymphs were found on any 35S::SAP66 so fecundity is unaffected by SAP66. These results indicate that SAP66 may play a significant role manipulating plant defence against non-host insects.



### Figure 5.2.2.1. SAP66 significantly affects *M. quadrilineatus* fecundity

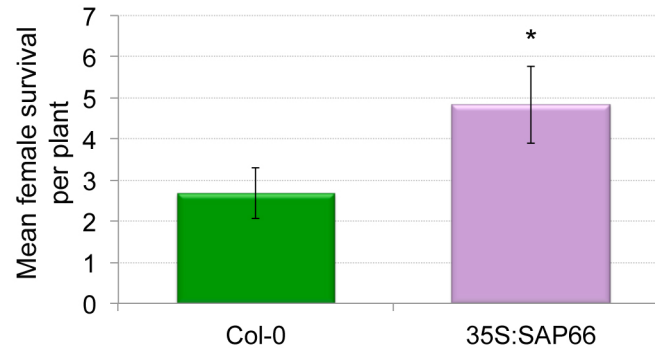
*M. quadrilineatus* fecundity was assessed on 35S:SAP66 Arabidopsis and control plants Col-0 and 35S:SAP11. Nymph production was significantly higher on 35S:SAP66,  $F_{3,27} = 3.2376$   $P = 0.04$ . Error bars highlight variability of insect performance as some 35S:SAP66 plants had over 40 nymphs, however most plants produced similar yield of nymphs as those on control Col-0 plants. SAP11 lines yielded higher nymph numbers than Col-0 as in previous experiments,  $F_{1,27} = 15.732$   $P = 0.01$ . Modeled by rep\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



### Figure 5.2.2.2. SAP66 merely has a significant effect on *M. quadrilineatus* fecundity when insects are challenged with CO<sub>2</sub> and cool conditions.

In these assays, 2 temperature conditions were included 22°C 10h day/14h night and 26°C h day/8h night. Four treatments were included, leafhoppers reared on Oats and treated with CO<sub>2</sub> and leafhoppers reared on Oats not treated with CO<sub>2</sub>, leafhoppers reared on Arabidopsis and treated with CO<sub>2</sub> and finally leafhoppers reared on Arabidopsis and not treated with CO<sub>2</sub>. *M. quadrilineatus* fecundity was tested on 3 35S:SAP66 lines with these treatments and condition together with Col-0 and 35S::SAP11 as control plants. Leafhoppers in experiments were 14-18 days old and 18-22 days old (means of plant lines and ages are shown). Insect age had no significant effect on *M. quadrilineatus* fecundity  $F_{1,109} = 37.651$   $P = 0.237$ . Fecundity was only significantly increased on 35S:SAP66 Arabidopsis when leafhoppers were reared on Arabidopsis (so had no host change) anesthetized with CO<sub>2</sub> and kept in cool conditions (22°C 10h L/ 14h D)  $F_{4,43} = 7.767$   $P = 0.01$ . This suggests that SAP66 has no biologically significant effect on *M. quadrilineatus* fecundity. SAP11 lines yielded higher nymph numbers than Col-0 in cool conditions, as in previous experiments,  $F_{1,57} = 15.732$   $P = 0.01$ . Modeled by rep\*treatment\*condition\*plant line\*age using GLM

with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.2.3. AY-WB secreted protein SAP66 increases *D. maidis* survival on Arabidopsis**

*D. maidis* survival on 35S:SAP66 was increased over Col-0 control plants,  $F_{1,36} = 3.748$   $P = 0.005$ . No egg or nymphs were found only on 35S:SAP66 plants, indicating fecundity is unaffected by SAP66. Modeled by rep\*plant line\*age using GLM with a binomial link function. Errors shown are 2x standard error above and below means.

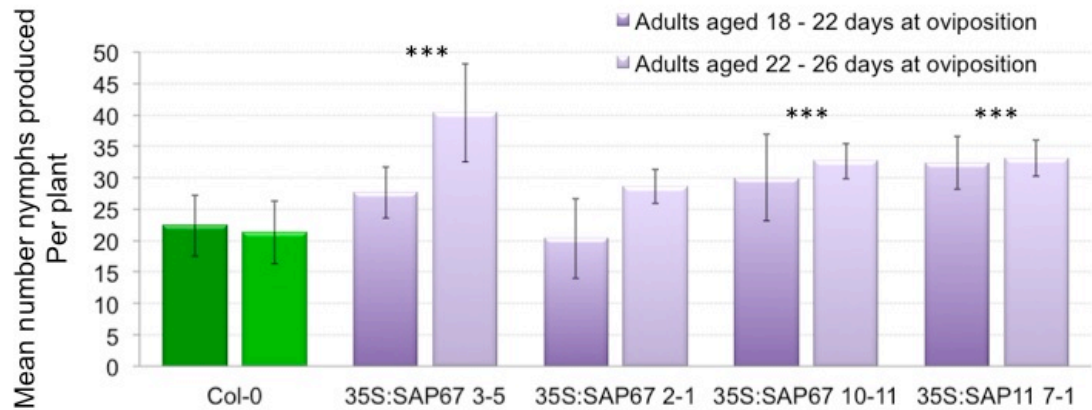
### 5.2.3 *M. quadrilineatus* has increased fecundity on SAP67 Arabidopsis

SAP67 expression in Arabidopsis has a significant increasing effect on *M. quadrilineatus* fecundity (Fig. 5.2.3.1). *M. quadrilineatus* produced over 60 nymphs on some 35S::SAP56 Arabidopsis and under 20 on others.

In further experiments with more controls, *M. quadrilineatus* fecundity was significantly increased by SAP67 in cool temperatures. Furthermore, this effect was increased further when insects were raised on oats, therefore insects were subjected to a host change at the start of the experiment. Interestingly, *M. quadrilineatus* have significantly higher fecundity on all plant lines in warmer longer day conditions (Fig. 5.2.3.2) that adds to the variability of nymph numbers and may mask the effect of SAP67 and SAP11 on *M. quadrilineatus* fecundity.

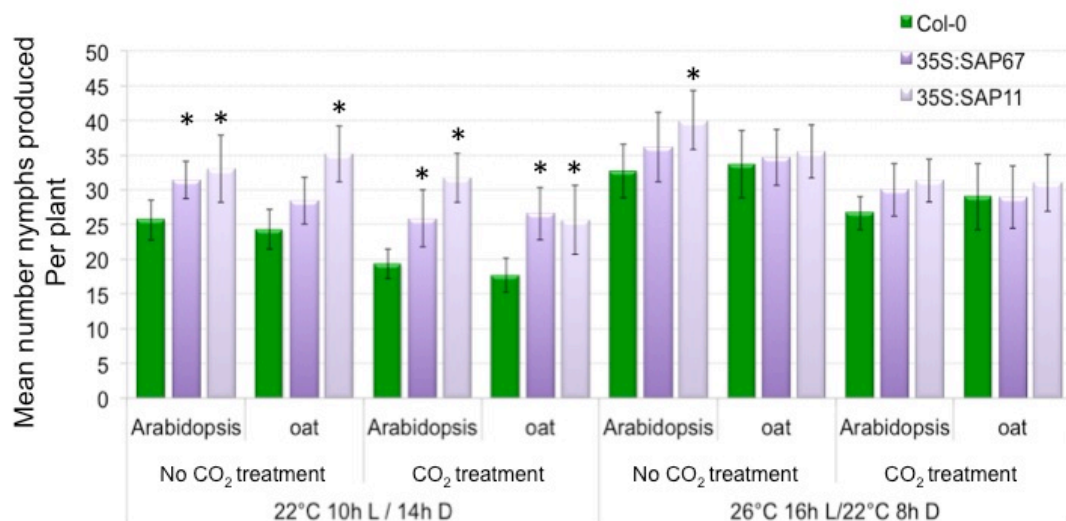
Survival of maize specialist *D. maidis* is unaffected by SAP67 in Arabidopsis,  $F_{1,19} = 16.2$   $P = 0.238$ . No eggs or nymphs were recorded on any 35S::SAP56 plants thus, fecundity is also unaffected by SAP67.

Therefore, SAP67 increases vector *M. quadrilineatus* fecundity when leafhoppers are in cool conditions, but has no effect on non-host interactions. Thus, it is likely that SAP67 plays a role in AY-WB manipulation of vector-plant interactions.



**Figure 5.2.3.1. SAP67 significantly increases *M. quadrilineatus* fecundity on Arabidopsis**

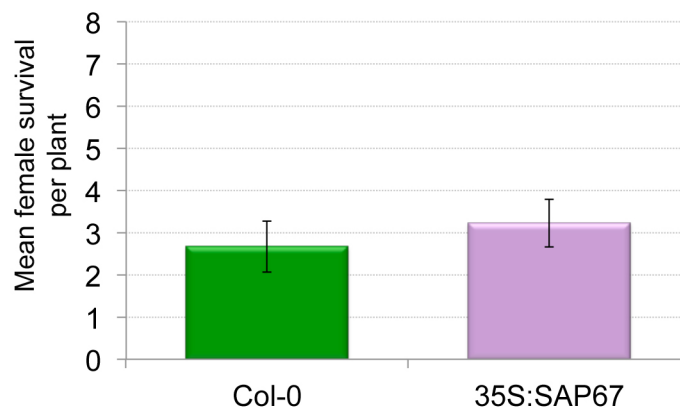
*M. quadrilineatus* nymph production was significantly different between Col-0 and 35S:SAP67,  $F_{3,36} = 17.682$   $P = 0.04$ . Columns and error bars highlight the variability of lines 35S:SAP67 3-5 and 35S:SAP67 2-1, and support re-examination using more effective control plants. Modeled by rep\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.3.2. SAP67 has a significant effect on *M. quadrilineatus* fecundity in cool conditions.**

In these assays, 2 temperature conditions were included 22°C 10h day/14h night and 26°C h day/8h night. Four treatments were included, leafhoppers reared on Oats and

treated with CO<sub>2</sub> and leafhoppers reared on Oats not treated with CO<sub>2</sub>, leafhoppers reared on Arabidopsis and treated with CO<sub>2</sub> and finally leafhoppers reared on Arabidopsis and not treated with CO<sub>2</sub>. *M. quadrilineatus* fecundity was tested on 3 35S:SAP67 lines with these treatments and condition together with Col-0 and 35S::SAP11 as control plants. Leafhoppers in experiments were 14-18 days old and 18-22 days old (means of plant lines and ages are shown). Insect age had no significant effect on *M. quadrilineatus* fecundity  $F_{1,104} = 23.448$   $P = 0.721$ . Leafhopper vectors showed significantly more nymph production on 35S:SAP67 Arabidopsis in cool conditions (22°C 10h L/ 14h D) compared with Col-0,  $F_{1,104} = 31.650$   $P = 0.013$ . This suggests that SAP67 and SAP11 have similar biologically significant effects on *M. quadrilineatus* fecundity. SAP11 lines yielded higher nymph numbers than Col-0 in cool conditions, as in previous experiments,  $F_{1,57} = 15.732$   $P = 0.01$ . Modeled by rep\*treatment\*condition\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.3.3. SAP67 has no significant effect on *D. maidis* survival on Arabidopsis**

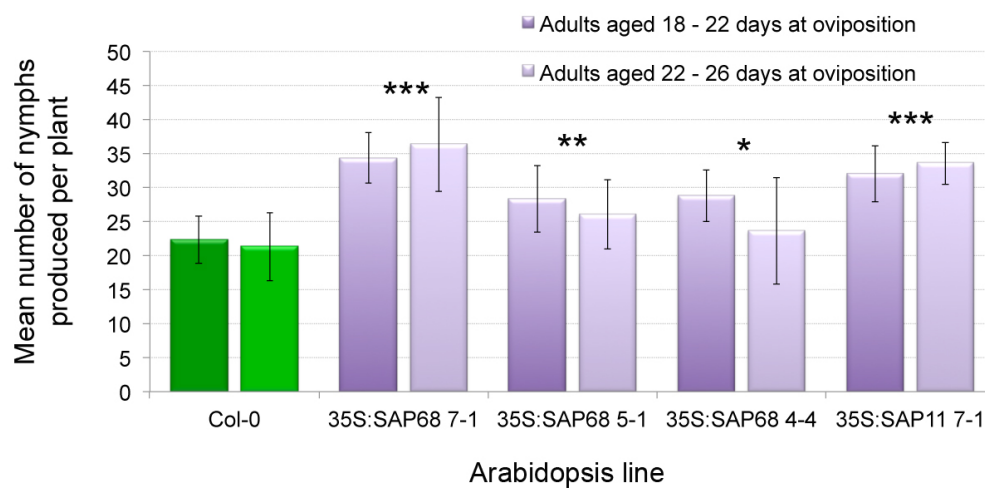
*D. maidis* survival on Arabidopsis line 35S:SAP67, was not significantly different from survival Col-0,  $F_{1,19} = 16.2$   $P = 0.238$ . No nymphs or eggs were found on 35S:SAP05 plants, therefore no difference in fecundity was observed on 35S:SAP67. Modeled by rep\*treatment\* plant line\*age using GLM with a binomial link function. Errors shown are 2x standard error above and below means.

#### 5.2.4 SAP68 has no significant effect on leafhopper-plant interactions

I found that SAP68 has no significant effect on *M. quadrilineatus* fecundity (Fig. 5.2.4.1). Outliers and made no significant impact to results but did affect variability. In addition, host change significantly

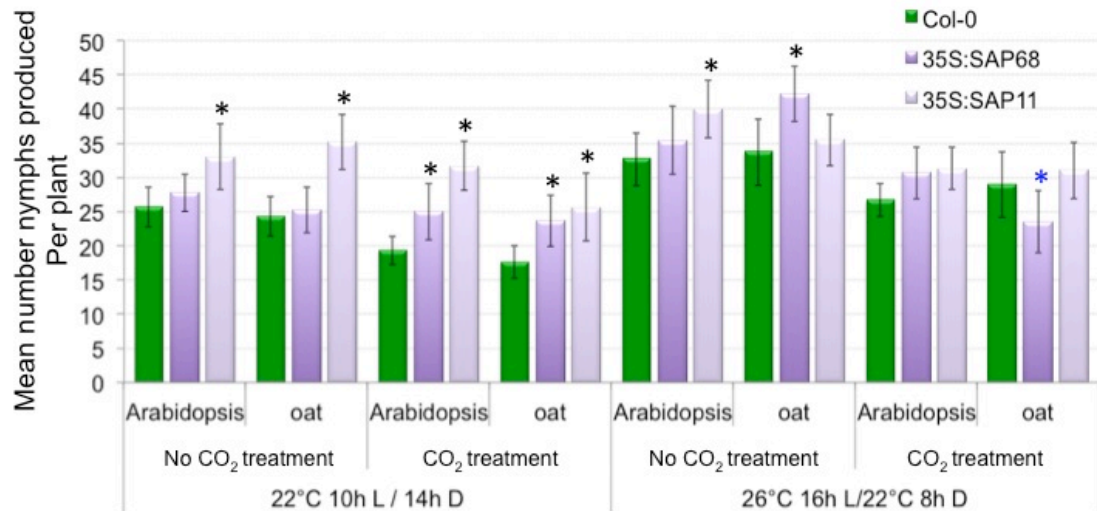
affected leafhopper fecundity in cooler conditions 35S:SAP68 Arabidopsis (Fig. 5.2.4.2). *M. quadrilineatus* fecundity was unaffected by SAP68 in any other condition.

Maize specialist leafhopper *D. maidis* survival is also unaffected by SAP68 expression in Arabidopsis  $F_{1,28} = 24.53$   $P = 0.431$  (Fig. 5.2.4.3). These results indicate that SAP68 may play a role with other proteins on the SAP11 PMU-like region in manipulating leafhopper-plant interactions.



**Figure 5.2.4.1. *M. quadrilineatus* fecundity is significantly increased on 35S::SAP68 Arabidopsis.**

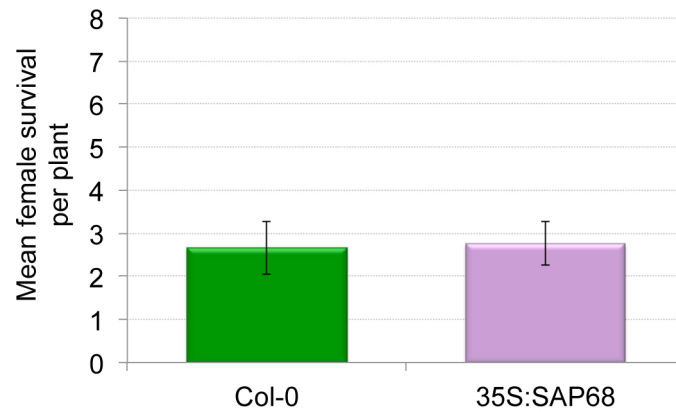
*M. quadrilineatus* fecundity was assessed on 35S:SAP68 Arabidopsis and Col-0 and 35S:SAP11 as controls. Nymphs production was significantly higher 335S:SAP68 and Col-0,  $F_{3,48} = 23.6502$   $P = 0.001$ . Error bars highlight the variability of the data and a few plants had over 50 nymphs, however many plants produced similar yield of nymphs as those on control Col-0 plants. SAP11 lines yielded higher nymph numbers than Col-0 as in previous experiments,  $F_{1,67} = 5.3108$   $P = <0.001$ . Modeled by rep\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.4.2. The increase in *M. quadrilineatus* fecundity on 35S::SAP68 is of limited significance.**

In these assays, 2 temperature conditions were included 22°C 10h day/14h night and 26°C h day/8h night. Four treatments were included, leafhoppers reared on Oats and treated with CO<sub>2</sub> and leafhoppers reared on Oats not treated with CO<sub>2</sub>, leafhoppers reared on Arabidopsis and treated with CO<sub>2</sub> and finally leafhoppers reared on Arabidopsis and not treated with CO<sub>2</sub>. *M. quadrilineatus* fecundity was tested on 3 35S:SAP68 lines with these treatments and condition together with Col-0 and 35S::SAP11 as control plants. Leafhoppers in experiments were 14-18 days old and 18-22 days old (means of plant lines and ages are shown). Insect age had no significant effect on *M. quadrilineatus* fecundity  $F_{1,111} = 16.5108$   $P = 0.531$ . Fecundity was only significantly increased on 35S:SAP68 Arabidopsis when leafhoppers were anesthetized with CO<sub>2</sub> and kept in cool conditions (22°C 10h L/ 14h D)  $F_{3,41} = 1.3075$   $P = 0.001$ . This suggests that the effect of SAP68 alone is of little biological significance, however a possible co-operative function with another protein may be worth investigation. SAP11 lines yielded higher nymph numbers than Col-0 in cool conditions, as in previous experiments,  $F_{1,57} = 15.732$   $P = 0.01$ . Modeled by  $\text{rep} \times \text{treatment} \times \text{condition} \times \text{plant line} \times \text{age}$  using GLM with a poisson link function. Errors shown are 2x standard error above and below means.





**Figure 5.2.4.3. SAP68 plays no role in manipulation of *D. maidis* survival on Arabidopsis**

*D. maidis* survival was not significantly different on 35S:SAP68 plants and Col-0,  $F_{1,28} = 24.53$   $P = 0.431$ . No eggs or nymphs were on any plants, thus no effect on fecundity was observed. . Modeled by rep\*treatment\*plant line\*age using GLM with a binomial link function. Errors shown are 2x standard error above and below means.

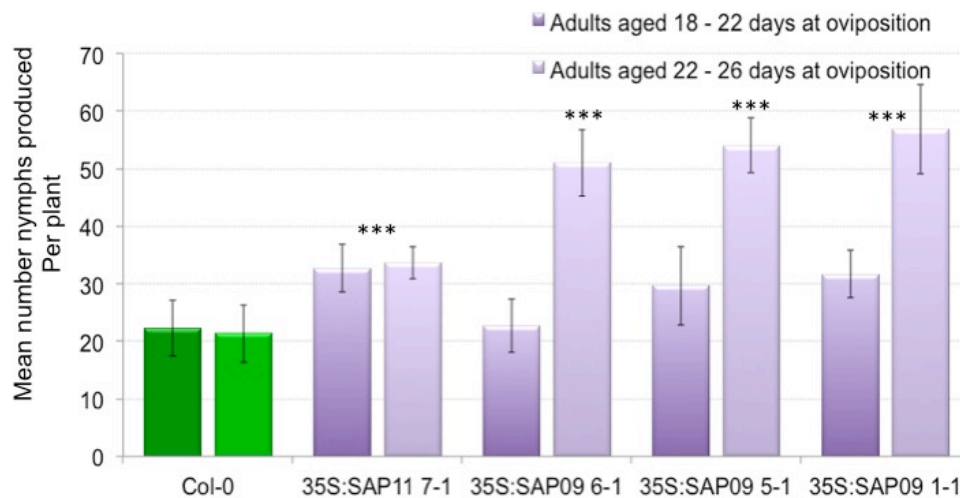
#### 5.2.5 SAP09 in Arabidopsis induces increased *M. quadrilineatus* oviposition

*M. quadrilineatus* fecundity is significantly increased on 35S:SAP09 Arabidopsis (Fig. 5.2.5.1)  $F_{2,38} = 33.692$   $P = 0.01$ . When leafhoppers are kept in cooler conditions, SAP09 significantly increases on *M. quadrilineatus* (Fig. 5.2.5.2). Host change also increases *M. quadrilineatus* on 35S:SAP09 in cool conditions. Anesthesia with CO<sub>2</sub> had no significant affect on fecundity. *M. quadrilineatus* have significantly higher fecundity in warmer longer day conditions (Fig. 5.2.5.2) confirming results in earlier chapters. The increase in fecundity on all Arabidopsis lines appears to mask the increase in fecundity seen on 35S::SAP11 and 35S::SAP09 (Fig. 5.2.5.2).

Interestingly, the clear effect on *M. quadrilineatus* is not translated to *D. maidis*. SAP09 had no significant effect on *D. maidis* survival  $F_{=1,24} = 78.335$   $P = 0.193$  (Fig. 5.2.5.3). No eggs or nymphs were found on any plants, therefore, *D. maidis* fecundity is unaffected by SAP56. These

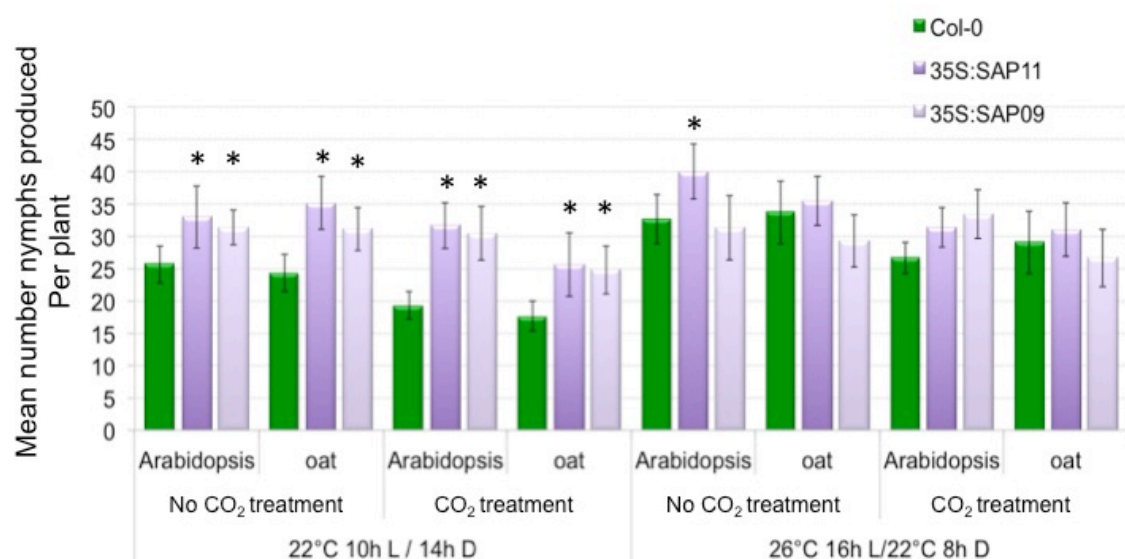


results indicate that SAP09 may play a role in AY-WB manipulation of vector-plant interactions.



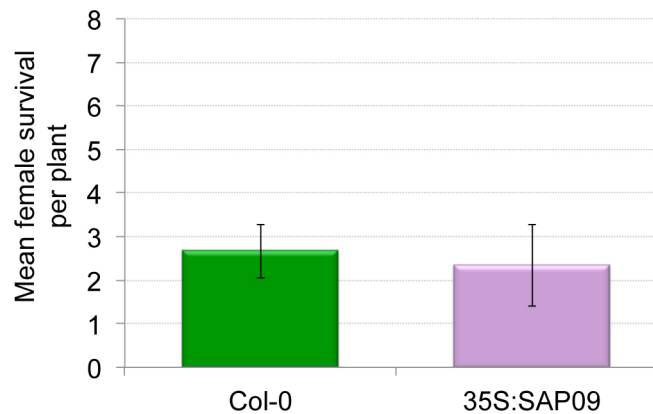
**Figure 5.2.5.1. SAP09 significantly increases *M. quadrilineatus* fecundity on Arabidopsis.**

*M. quadrilineatus* fecundity was assessed on 35S:SAP09 Arabidopsis and Col-0 and 35S:SAP11 as controls. Nymphs production was significantly higher 335S:SAP09 and Col-0,  $F_{2,51} = 13.751$   $P = 0.021$ . Columns and error bars highlight the variability of the data and a few plants had over 60 nymphs, however many plants produced similar yield of nymphs as those on control Col-0 plants. SAP11 lines yielded higher nymph numbers than Col-0 as in previous experiments,  $F_{1,67} = 5.3108$   $P = <0.001$ . Modeled by rep\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.5.2. SAP09 has a significant effect on *M. quadrilineatus* fecundity in cool conditions.**

In these assays, 2 temperature conditions were included 22°C 10h day/14h night and 26°C 10h day/8h night. Four treatments were included, leafhoppers reared on Oats and treated with CO<sub>2</sub> and leafhoppers reared on Oats not treated with CO<sub>2</sub>, leafhoppers reared on Arabidopsis and treated with CO<sub>2</sub> and finally leafhoppers reared on Arabidopsis and not treated with CO<sub>2</sub>. *M. quadrilineatus* fecundity was tested on 3 35S:SAP09 lines with these treatments and condition together with Col-0 and 35S::SAP11 as control plants. Leafhoppers in experiments were 14-18 days old and 18-22 days old (means of plant lines and ages are shown). Insect age had no significant effect on *M. quadrilineatus* fecundity  $F_{1,106} = 15.411$   $P = 0.914$ . Fecundity was only significantly increased on 35S:SAP09 Arabidopsis when experiments were conducted in cool conditions (22°C 10h L/ 14h D)  $F_{1,106} = 17.068$   $P = <0.001$ . This suggests that SAP09 and SAP11 have similar levels of biologically significant effects on *M. quadrilineatus* fecundity. SAP11 lines yielded higher nymph numbers than Col-0 in cool conditions, as in previous experiments,  $F_{1,57} = 15.732$   $P = 0.01$ . Modeled by rep\*treatment\*condition\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.5.3. *D. maidis* survival is unaffected by SAP09 accumulation in Arabidopsis.**

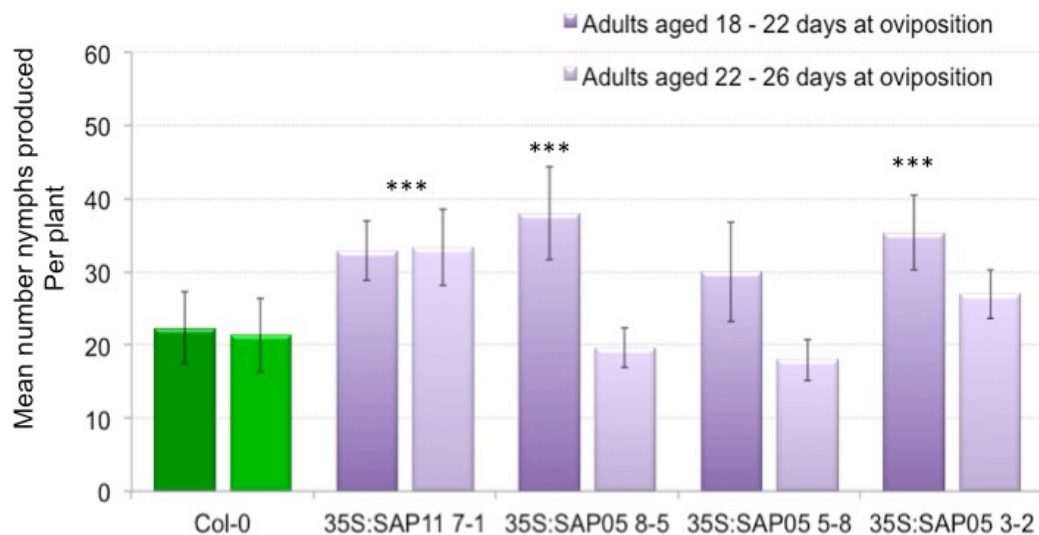
*D. maidis* survival on 35S:SAP09 Arabidopsis is not significantly different from control Col-0 plants,  $F_{1,24} = 78.335$   $P = 0.193$ . No eggs or nymphs were on any plant, therefore no increase in fecundity was observed. Modeled by rep\*treatment\*plant line\*age using GLM with a binomial link function. Errors shown are 2x standard error above and below means.

### 5.2.6 SAP05 in *Arabidopsis* increases fitness of *M. quadrilineatus* and *D. maidis*.

*M. quadrilineatus* fecundity is significantly increased on lines 35S:SAP05 8-5 and 35S::SAP05 3-2 compared with Col-0 controls (Fig. 5.2.6.1). However, columns and error bars indicate that this is highly variable and this difference requires re-examination.

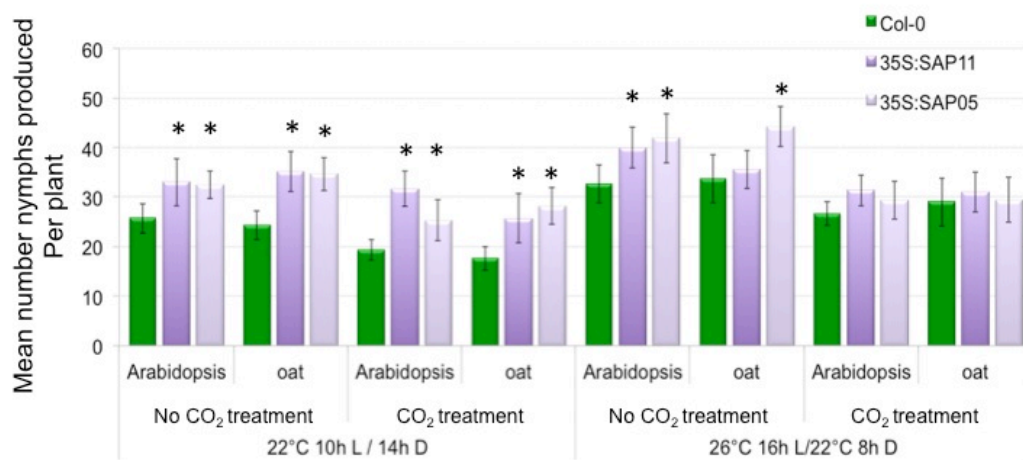
In repeated experiments that control for environmental and experimental design impacts, a clearer indication of SAP05 significance was obtained. When experiments are conducted in cooler conditions, SAP05 significantly increases on *M. quadrilineatus* (Fig. 5.2.6.2). Host change does not significantly affect *M. quadrilineatus* on 35S:SAP05 in cool conditions. Anesthesia with CO<sub>2</sub> also had a significant affect on fecundity in warmer conditions.

Interestingly, *D. maidis* survival on 35S::SAP05 is also significantly increased compared with Col-0,  $F_{1,36} = 7.4078$   $P = <0.001$  (Fig. 5.2.6.3). In addition, *D. maidis* oviposited on 35S::SAP05 lines; 5 eggs and one nymph were found on 4 35S:SAP05 plants indicating SAP05 may also play a role in manipulating *D. maidis* fecundity on AY-WB infected *Arabidopsis*.



**Figure 5.2.6.1. SAP05 has a variable but significant increasing effect on *M. quadrilineatus* fecundity**

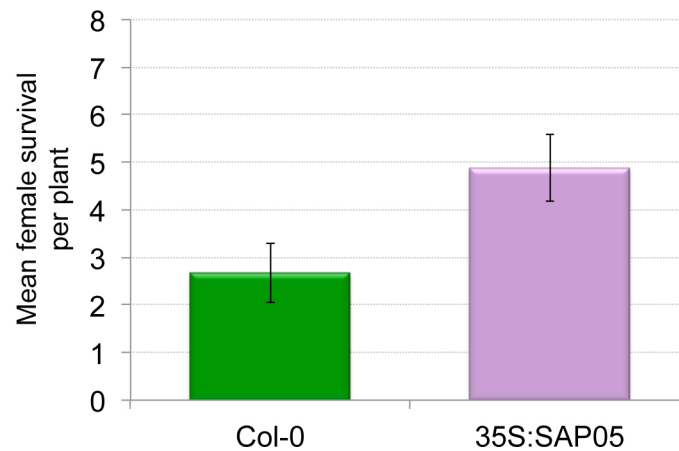
*M. quadrilineatus* fecundity was assessed on 35S:SAP05 Arabidopsis and Col-0 and 35S:SAP11 as controls. Nymphs production was significantly higher 35S:SAP05 and Col-0,  $F_{2,58} = 7.4635$   $P = 0.034$ . Columns and error bars highlight the variability of the data and a few plants had over 45 nymphs, however many plants produced similar or less nymphs as those on control Col-0 plants. SAP11 lines yielded higher nymph numbers than Col-0 as in previous experiments,  $F_{1,67} = 5.3108$   $P = <0.001$ . Modeled by rep\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.6.2. SAP05 has a significant effect on *M. quadrilineatus* fecundity when experiments are conducted in cool conditions and when insects are not treated with CO<sub>2</sub>.**

In these assays, 2 temperature conditions were included 22°C 10h day/14h night and 26°C h day/8h night. Four treatments were included, leafhoppers reared on Oats and treated with CO<sub>2</sub> and leafhoppers reared on Oats not treated with CO<sub>2</sub>, leafhoppers reared on Arabidopsis and treated with CO<sub>2</sub> and finally leafhoppers reared on Arabidopsis and not treated with CO<sub>2</sub>. *M. quadrilineatus* fecundity was tested on 3 35S:SAP05 lines with these treatments and conditions together with Col-0 and 35S::SAP11 as control plants. Leafhoppers in experiments were 14-18 days old and 18-22 days old (means of plant lines and ages are shown). Insect age had no significant effect on *M. quadrilineatus* fecundity  $F_{1,114} = 26.193$   $P = 0.105$ . Fecundity significantly increased on 35S:SAP05 Arabidopsis when experiments were conducted cool conditions (22°C 10h L/ 14h D)  $F_{1,114} = 9.851$   $P = 0.026$ . Fecundity was also increased on 35S:SAP05 Arabidopsis in warm conditions when leafhoppers were not treated with CO<sub>2</sub>  $F_{3,52} = 37.408$ ,  $P = 0.001$ . This suggests that SAP05 has a biologically significant effect on *M. quadrilineatus* fecundity. SAP11 lines yielded higher nymph numbers than Col-0 in cool conditions, as in previous experiments,  $F_{1,57} = 15.732$   $P =$

0.01. Modeled by rep\*treatment\*condition\*plant line\*age using GLM with a poisson link function. Errors shown are 2x standard error above and below means.



**Figure 5.2.6.3. *D. maidis* survival is unaffected by SAP05 in Arabidopsis**

*D. maidis* survival on 35S:SAP09 Arabidopsis is not significantly different from control Col-0 plants,  $F_{1,24} = 78.335$ ,  $P = 0.193$ . No eggs or nymphs were on any plant, therefore no increase in fecundity was observed. Data analysed with analysis of deviance in Genstat v.13.

## Discussion

### 5.3.1 SAP05 increases fitness of both *D. maidis* and *M. quadrilineatus*

Stable expression of AY-WB secreted protein, SAP05 in Arabidopsis generates heart shaped leaves and earlier bolting (data from Hogenhout, unpublished). *M. quadrilineatus* fecundity is increased by 21% on 35S:SAP05 Arabidopsis indicating that SAP05 plays a part in adjusting Arabidopsis to be a more suitable host to the leafhopper. Furthermore, maize specialist leafhopper *D. maidis* has increased survival on 35S:SAP05 Arabidopsis and oviposits on this non-host plant. This suggests that whilst the phenotypic changes caused by SAP05 are mild, insect-plant interactions both host and non-host are significantly modulated. Further examination of SAP05-plant interactions are required to gain clearer understanding of the function of SAP05. As this candidate secreted protein appears to have an effect on both *D. maidis* survival and *M. quadrilineatus* fecundity in these

preliminary studies, I would suggest this would be a suitable next AY-WB candidate secreted protein to study further.

### 5.3.2 SAP56, 66, 67, 68 and 09 are candidates for cross experiments

*M. quadrilineatus* fecundity increase on 35S:SAP56 and 35S:SAP66 Arabidopsis plants is only significant in one contrived lab condition (Fig. 5.2.1.2 and Fig 5.2.2.2). However, *D. maidis* survival is increased on 35S:SAP56 Arabidopsis plants by 34% and on 35S:SAP66 Arabidopsis by 37%. Results here indicate that AY-WB candidate secreted proteins SAP56 and SAP66 warrant more examination in relation to their impact on non-host insect-plant interactions rather than vector-plant interactions. They could also be further studied in Arabidopsis crossed with other 35S:SAP lines to examine effects of multiple secreted proteins on insect-plant interactions.

35S:SAP67 and 35S:SAP68 Arabidopsis induced significantly increased *M. quadrilineatus* nymph production in cool conditions (Fig 5.2.3.2 and Fig. 5.2.4.2). *D. maidis* survival showed no significant increase on either 35S:SAP67 and 35S:SAP68 Arabidopsis. These data suggest that *M. quadrilineatus*-Arabidopsis interactions may be modulated by SAP67 and SAP68. Further examination of these 35S:SAP67x35S:SAP68 and or crosses with 35S:SAP11 may have additive effects on insect fitness or highlight that these proteins interact within the plant to alter *M. quadrilineatus* fitness.

Nymph production of *M. quadrilineatus* leafhoppers was significantly increased on 35S:SAP09 Arabidopsis in cool and warm conditions (Fig. 5.2.5.2). These data indicate that further examination of the role of SAP09 in modulating insect-plant interactions needs to be explored. *D. maidis* survival remained unchanged on 35S:SAP09 which suggests that SAP09 plays a role in Arabidopsis that irrelevant to *D. maidis* and possibly other non-host insect herbivores.

Investigations into the roles of SAP09, SAP67 and SAP68 in *M. quadrilineatus* interactions with Arabidopsis, either alone or in

combination would provide insight into the function of these 3 proteins and their parts in plant host manipulation. Similar study of SAP66 and SAP56 in conjunction with *D. maidis* may provide useful information in non-host and novel insect herbivore interactions with Arabidopsis, alone or in crosses.

Conducting yeast two hybrid screens to find interactors in Arabidopsis would be a great next step to assess the role of these proteins. I would suggest SAP05 and SAP09 are good candidate proteins to examine first.

### *5.3.3 Impacts of environmental factors indicated the variability in efficacy of SAP manipulations.*

All secreted effector proteins investigated in this chapter only exhibited effects in certain conditions, including SAP11 controls. Most notable conditions where effects were significant were in cool conditions 22°C 10h L/ 14h D. It is probable that AY-WB phytoplasma transmission is affected by environmental conditions and the limited significance of these SAPs may be evidence of this. Vector insects of AYPs are shown to have reduced efficacy in transmission of phytoplasmas in prolonged high temperatures (Goodwin *et al.* 1999). Other bacteria such as symbionts found in insects are known to suffer as a result of high temperatures and which affect insect immune systems (Schmid-Hempel 2004). Examining AY-WB transmission and efficacy at manipulation of hosts in various environmental conditions yields clues as to the limits of phytoplasma dispersal and potential control strategies. It is plausible that AY-WB secreted effector proteins may have reduced efficacy in high temperatures which in turn effects AY-WB success.

## Discussion

*D.1 Arabidopsis thaliana is a good model plant to use when studying phytoplasma manipulations of host plants and their interactions.*

*Arabidopsis thaliana* is found in the same geographic locations as *M. quadrilineatus* across USA and Canada. *Arabidopsis* ecotype Col-0 is host to both *M. quadrilineatus* and AY-WB phytoplasmas in lab conditions. Furthermore, symptoms of AY-WB in *Arabidopsis* Col-0 are reproducible when 5 week old plants are inoculated with 3 AY-WB infected male *M. quadrilineatus* and the plants are grown in short day conditions at 22°C consistently with a 10 hour day and 14 hour night. These conditions delay plant bolting, which is induced by increased temperature and day length, but also minimize the impacts of phytoplasma load and herbivore activity on the young plants, which can be lethal. Reproducible uniform plants, provide plants for many experiments to investigate AY-WB effects on host plants.

*M. quadrilineatus* fecundity is increased on AY-WB infected *Arabidopsis* which aligns with findings of others on phytoplasma infected lettuce and aster (Barnes 1954, Purcell 1973). In addition, the numbers of infected and uninfected *Arabidopsis* that can be produced in a short timeframe enabled experiments to distinguish the effect of AY-WB in the insect from the effect of AY-WB in the plant on insect-plant interactions. This has not been previously examined and could yield valuable clues as the dispersal and epidemiology of phytoplasmas

*Arabidopsis* model plants also provide a vast molecular tool kit that can be used to study phytoplasma manipulation directly in a host plant with a short generation time. For example, a microarray study of *Arabidopsis* genes 1 and 2 weeks post AY-WB infection, revealed that only 5 *Arabidopsis* genes had altered expression 1 week after infection compared with >1000 gene 2 weeks after infection. Thus indicating that AY-WB phytoplasmas alters gene expression in their host plant. Use of *Arabidopsis thaliana* allows for in depth study of these changes in gene expression that is not possible in field crops. Using this fundamental



approach will translate to further avenues of targeted study of phytoplasma manipulations of crop host plants.

An interesting future test of *Arabidopsis* as a model host plant for phytoplasma study, would be to examine infection levels and symptoms of other fully sequenced phytoplasmas in *Arabidopsis*. Assuming that phytoplasmas are limited in spreading to new plant hosts by the host plant choice of their vectors, it is theoretically possible that all phytoplasmas could infect *Arabidopsis* if their vectors can feed on the plant. Thus, if insect vectors are enclosed on *Arabidopsis* plants, having only the choice to feed or die, the phytoplasma could be transmitted to the novel *Arabidopsis* plants. Vector efficacy would have to be measured and added as a variable to a model in analysis. Onion yellows phytoplasma, and *Ca. phytoplasma australiense* are all fully sequenced and would provide two phytoplasmas with diverse plant host ranges for this experiment. Furthermore, the distributions of these phytoplasmas do not overlay with the distribution of *Arabidopsis*, therefore neither would ordinarily come into contact with *Arabidopsis*. Onion yellows, has a similar large plant host range to that of AY-WB and is an AYP within subgroup 16SrI-B. In addition, its vector *Macrostoteles striifrons* is a polyphagous leafhopper of Asia, and in the same genus as *M. quadrilineatus* (though not the same species complex). *Ca. phytoplasma australiense* is a stolbur phytoplasma in group 16SrXII and endemic to New Zealand and Australia. *Phytoplasma australiense* has a large host range including strawberries (*Fragaria ananassa*), potatoes (*Solanum tuberosum*), New Zealand flax lily (*Phormium tenax*), grape vines (*Vitis vinifera*), the Karamu shrub in New Zealand (*Coprosoma robusta*) and the cabbage tree (*Cordyline australis*) (Maixner 2008, Beever *et al.* 2004, Saquib *et al.* 2006, Liefting *et al.* 2009) and. Two known vectors that could be used in experiments are planthoppers *Zeoliarus atkinsoni* to *Phormium tenax* in New Zealand, and the polyphagous *Z. oppositus* to *Coprosoma robusta* and others potentially. Other endemic leafhoppers and planthoppers in New Zealand and Australia are under investigation as potential vectors of *Ca.*

phytoplasma australiense to other plants (Beever *et al.* 2008, Liefting *et al.* 2011). Studying the transmission level, spread of infection and symptom development of these two phytoplasmas in Arabidopsis, in comparison with AY-WB, could yield fundamental clues as to how phytoplasmas manipulate naïve plant hosts. In addition, examining similarities and difference in Arabidopsis gene expression in response to phytoplasma infection could yield further clues as to the mechanisms of phytoplasma manipulation of host plants and differences in virulence.

*D.2 AY-WB infection within Arabidopsis thaliana increases the susceptibility of the plants to insect herbivores.*

*M. quadrilineatus* has similar survival but produces more nymphs on AY-WB infected Arabidopsis compared with numbers on uninfected Arabidopsis plants. These data support those recorded in other study plants such as Aster and Lettuce (Beanland *et al.* 2000; Ebbert and Nault 1998). In these previous studies all insects and plants in experiments were infected with aster yellows phytoplasmas. Thus, it is impossible to ascertain the origin of any effects on leafhopper survival or fecundity. This study examines leafhopper survival fecundity and behaviour when only the plant, or the insects are infected, and compares these results with those in which both or neither are infected. This is the first study that separates effects of phytoplasma infection within the plant and infection within the insect, on insect-plant interactions. The separation is essential in understanding how phytoplasmas manipulate insect hosts and plant hosts and therefore their interactions.

Survival of *M. quadrilineatus* was not significantly different on AY-WB infected Arabidopsis over uninfected, however this was only measured over a 4day period. Thus, this did not take into account any longer term mortality effects on survival of the insects, which are accounted for in

other studies. Beanland *et al.* found that *M. quadrilineatus* survived significantly longer on AYP 'bolt' infected and APY 'severe' infected aster and lettuce than on uninfected plants. However there was no significant difference in the first 5 days of the experiments. Short-term experiments over 4 days enable surety that only insects or plants are infected with the phytoplasma and not both. Therefore I decided a trade off in favour of separation of effects in the insects and plants was more desirable than examining longer term mortality. Another way to examine long-term effects without compromising separation of effects would be to use the same insects throughout the experiment but move them to new plants every 4 days. However in doing this, insects not placed on new plants every 4 days would need to be controlled for and included as a factor in the model during analysis. Changing of plant host once may cause an effect on survival and fecundity of insects. Changing the plant multiple times over the insect life span may have additive effects that could generate significant noise in the data, that would need to be properly controlled for. This would require unrealistic growth room space for experimental and control plants.

AY-WB phytoplasma alters defence capabilities in Arabidopsis and changes plant morphology, which in turn render plants more attractive or suitable to insects. Furthermore, AY-WB altered plant height, colour, leaf proliferation and greening of flowers probably render infected plants more obvious or attractive to many herbivorous insects. I demonstrate that *M. quadrilineatus* visited significantly more AY-WB infected Arabidopsis than uninfected plants (Fig. 1.2.6.1), reinforcing the theory.

*M. quadrilineatus* feeding is significantly lower on AY-WB infected Arabidopsis (Fig. 1.2.5.1) than uninfected Arabidopsis. This indicates that *M. quadrilineatus* may find it easier to feed on AY-WB infected Arabidopsis, or need to feed less. This may be due to changes in nutrient uptake from infected phloem, or suppressed plant defence interruption of feeding. Some phytoplasmas are shown to alter the carbohydrate concentrations in leaf phloem (Lepka *et al.* 1999; Maust *et*

al. 2003). Therefore it is plausible that AY-WB alters leaf phloem sugar concentrations, resulting in reduced feeding by *M. quadrilineatus*. Similarly, AY-WB secreted protein SAP11 reduces the defence response of Arabidopsis. Which may reduce interruption of leafhopper feeding, resulting in efficient feeding by *M. quadrilineatus*. Further study of leafhopper of leaf phloem carbohydrate levels in AY-WB infected Arabidopsis and analysis of feeding using electropenetrationgraphs, would be appropriate to determine whether one or both of these factors have influence over *M. quadrilineatus* feeding levels.

AY-WB infection in Arabidopsis may also modulate interactions with non-host leafhoppers. *Dalbulus maidis*, a specialist leafhopper on maize has significantly increased survival on AY-WB infected Arabidopsis over uninfected Arabidopsis.

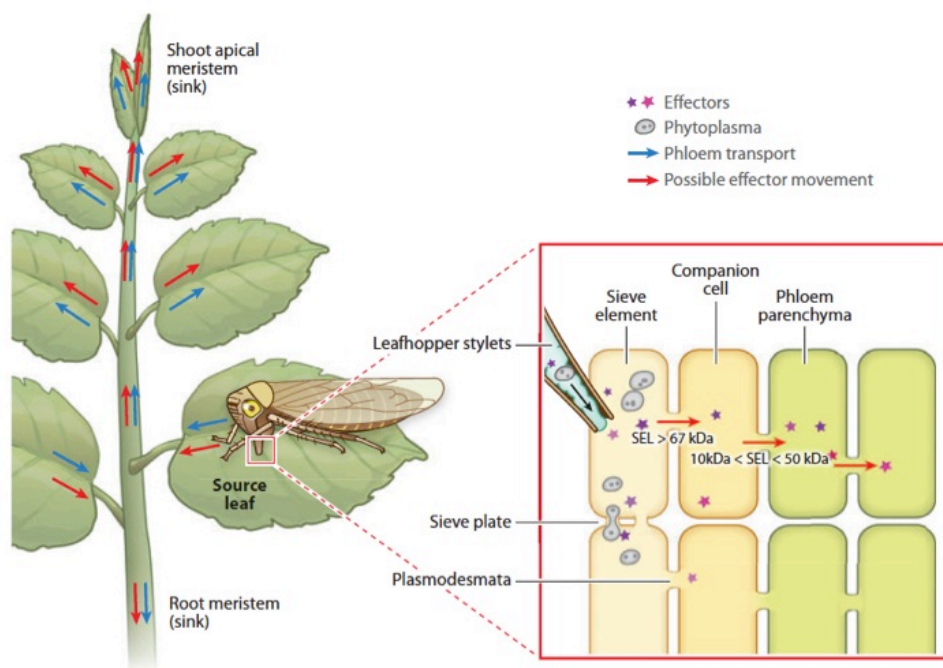
Survival of *D. maidis* on non-host Arabidopsis is low at 32% in females and 25% in males when enclosed on plants for 4 days. In contrast, *D. maidis* survival on AY-WB infected Arabidopsis increases to 54% and 48% for females and males respectively. In addition, *D. maidis* eggs were found on a few AY-WB infected plants, indicating that AY-WB in Arabidopsis also renders the plant suitable to *D. maidis* oviposition. *D. maidis* egg hatch rate was 35%, similar to that found on non-host grasses when leafhoppers are restricted to these non-host plants (Pitre, 1970). These data support the hypothesis that when this leafhopper has access to only non-host plants it will try to feed and reproduce (Nault, 1994). Almost all eggs were found hidden on the underside of mature Arabidopsis leaves along the lower mid vein. On host maize plants eggs are oviposited along veins on the inside of leaf blades close to the main stem. This indicates the leafhoppers locate areas plants where their eggs are hidden, perhaps from potential egg parasitoids.

*D. maidis* appeared to feed more easily on AY-WB infected Arabidopsis over uninfected. The over all quantity of feed/probe sites was not different but the damage to cells made at these sites were less

pronounced on AY-WB infected Arabidopsis. Feed/probe sites were predominantly on open tissue and tertiary veins of the leaves, but this behaviour was unaltered between healthy and AY-WB infected leaves. This is not surprising as the architecture of veins on maize leaves is considerably different to that on Arabidopsis leaves. The location of *D. maidis* feed/probe sites was indicative of inefficient feeding on a non-familiar host. AY-WB manipulation of *D. maidis* survival and fecundity on non-host Arabidopsis provides a model system in which to investigate how phytoplasmas recruit potential vectors.

### *D.3 AY-WB secreted proteins play a part in modulating plant responses to insect herbivory.*

AY-WB has 56 secreted proteins, 7 of which, included in this study, exhibit manipulations of Arabidopsis that in turn affect insect plant interactions. Secreted effector proteins target processes in the plant, inducing changes that facilitate the altered insect responses to plants and increased phytoplasma dispersal. Proteins must be unloaded from phloem where phytoplasmas are confined to have an effect on host plants (Fig. D.3.1).



**Figure D.3.1 AY-WB effector proteins such as SAP11 unload from phloem and move systemically in plants to alter plant cell transcription and plant morphology.**

Figure taken from Sugio *et al.* 2011b, made by John Innes Centre, Norwich, UK. Phytoplasmas move systemically through the plant and effector proteins are secreted from phytoplasma around the plant, where they induce changes in host plants.

The AY-WB secreted effector protein SAP11 destabilizes Arabidopsis TCPs and downstream synthesis of LOX2 and jasmonic acid (Sugio *et al.* 2011). *M. quadrilineatus* has increased fecundity on Arabidopsis with stable expression of 35S:SAP11. Therefore, AY-WB secreted SAP11 plays a part in rendering Arabidopsis more susceptible to herbivory or oviposition of *M. quadrilineatus*. Feeding on 35S:SAP11 plants is not significantly altered compared with uninfected. This suggests that SAP11 may not affect *M. quadrilineatus* feeding but may affect suitability of the plant for oviposition. *M. quadrilineatus* also has increased fecundity on LOX2 deficient and TCP deficient Arabidopsis. Thus, giving confirmation of the mode of action and contribution of SAP11 in AY-WB infected Arabidopsis. AY-WB uses the secreted effector protein SAP11 to modulate host plant defence via destabilization of TCPs and jasmonic acid synthesis. Thus, SAP11 plays a clear role in manipulation of plant host defence and an indirect role (via the plant) in increasing *M. quadrilineatus* fecundity.

I found that in expression of SAP11 made no difference to the survival of *D. maidis* and no eggs or nymphs were recorded. The compromise of LOX2, although shown to increase attraction of novel herbivores to *Nicotiana attenuata* (Kessler *et al.* 2004), had no effect on the survival of *D. maidis* and no eggs or nymphs were recorded. Interestingly, *D. maidis* survival was increased on Jar-1 Arabidopsis with compromised production of jasmonic acid, although no eggs or nymphs were recorded on these plants. Whilst SAP11 destabilizes TCPs and jasmonic acid production, reducing plant defence to *M. quadrilineatus*, *D. maidis* is unaffected by SAP11 manipulations of non-host Arabidopsis. This

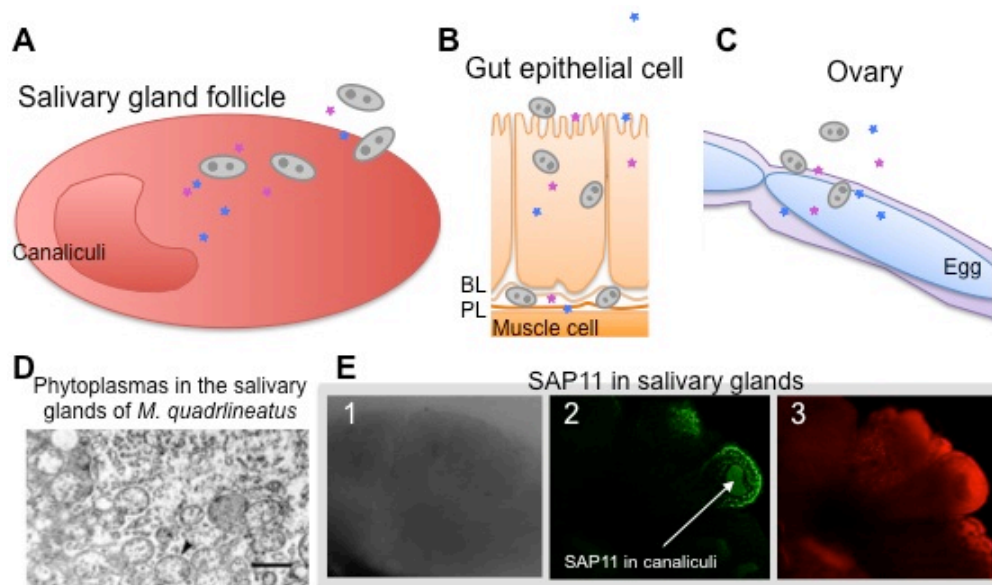
indicates that other SAPs may play a part in enabling *D. maidis* to survive and reproduce on non-host Arabidopsis.

Stable expression of AY-WB secreted protein, SAP05 in Arabidopsis generates heart shaped leaves and earlier bolting. *M. quadrilineatus* fecundity is increased by SAP05 indicating that SAP05 plays a part in adjusting Arabidopsis to be a more suitable host to the leafhopper. Furthermore, maize specialist leafhopper *D. maidis* has increased survival on 35S:SAP05 Arabidopsis although oviposition was only recorded on a few plants. This suggests that both host and non-host are manipulated.

*M. quadrilineatus* has unchanged fecundity on 35S:SAP56 or 35S:SAP66 Arabidopsis, however, *D. maidis* survival is increased on both these lines. 35S:SAP67 and 35S:SAP68 Arabidopsis induced increased *M. quadrilineatus* only in cool conditions and had no significant effect on *D. maidis* survival. Whilst *M. quadrilineatus* fecundity was increased on 35S:SAP09 Arabidopsis, *D. maidis* survival remained unchanged. Further investigation into the roles of these proteins would provide more information on AY-WB secreted effector proteins and their parts in plant host manipulation.

The secreted effector protein SAP11 is found throughout Arabidopsis one week after infection and in salivary glands of *M. quadrilineatus* 14 days after infection (Fig. D.5.3). SAP11 may be secreted in saliva separately to AY-WB, contributing to AY-WB effect on plants moments after infection. However, SAP11 may play a role in transmission of phytoplasmas from the salivary glands as it has not been found in guts or haemolymph of *M. quadrilineatus* and not looked for in ovaries. Further localization of SAP11 in *M. quadrilineatus* may reveal that its role is primarily in host plants. AY-WB secreted effector proteins translated with SAP11 could be localized in insect salivary glands and in plants at very early stages of infection. Antibodies to these proteins could be used to localize them in insects and in Arabidopsis over stages of infection. This will help to determine if and how AY-WB manipulates

vector, plant or both hosts to increase infected *M. quadrilineatus* oviposition.



**D.3.2. AY-WB phytoplasma are found throughout *M. quadrilineatus* and SAP11 has been found in the salivary glands.** AY-WB and SAP11 is found in the salivary glands of *M. quadrilineatus* (A, D, and E). It is likely that AY-WB utilizes secreted effector proteins in invading insect gut epithelial cells (B) and ovaries and eggs (C). AY-WB in salivary gland photo (D), adapted from Hogenhout *et al.* 2008. SAP11 is clearly visible in the canaliculi of a salivary gland follicle of an AY-WB infected *M. quadrilineatus* (E2). Confocal microscopy images highlight bright field (E1), green alexa fluor fluorescence excitation (E2) and red propidium iodide fluorescence excitation (E3) of salivary glands prepared by L. Landi and H. Kingdom. Localizing secreted effector proteins in *M. quadrilineatus* organs will yield clues as to the function of AY-WB effectors in insects.

**D.4** *AY-WB infection within the insect increases the fecundity of *M. quadrilineatus*.*

*M. quadrilineatus* have increased oviposition on AY-WB infected Arabidopsis (Fig. 1.2.3.2 [Chpt1]). Interestingly, AY-WB infected vector females have increased oviposition on uninfected Arabidopsis after only 2 days on uninfected plants (Fig. 2.2.1.2). Indicating that AY-WB is



manipulating the plant immediately after initial infection, or that the female insects are manipulated from within.

AY-WB accumulation in plant sink tissues occurs one week after initial infection (Ammar *et al* 2006; Hogenhout *et al.* 2008). AY-WB secreted protein SAP11, responsible for manipulation of plant defence, is found throughout Arabidopsis seedling roots, one week after infection (data from Hogenhout lab, not shown). In microarray data, AY-WB infection in Arabidopsis showed minimal transcriptional changes in the plant, whereas, 2 weeks after infection transcriptional changes were highly significant (data from Hogenhout lab, not shown). It is unclear to what degree AY-WB can affect plant hosts immediately after initial infection or one day post infection. Thus, it is plausible that increased infected *M. quadrilineatus* fecundity stems from within the insect alone or from within both hosts.

Within the insect, AY-WB is found in the salivary glands, saliva, gut, haemolymph and ovaries 10 days post infection (Fig. 2.2.4.1 and 2.2.4.2). In fecundity assays, AY-WB infected insects were raised on AY-WB infected plants, thus insects had an exposure of at least 3 weeks to AY-WB prior to experiments (through nymph development and eclosure). AY-WB proliferation to insect organs after 10 days, suggests that AY-WB and secreted proteins have 10 days to accumulate and affect insect biological processes. In experimental insects, this accumulation time is increased to 21 days, thus, a more pronounced effect might occur.

AY-WB within the ovaries may affect oviposition levels, egg maturity or egg fitness. It is likely that AY-WB affects Arabidopsis and *M. quadrilineatus* in their interactions. Further experiments to assess AY-WB manipulations within the insect and within Arabidopsis at initial and early infection, will yield clues as to the root of increased vector fecundity on healthy plants.

I have demonstrated that AY-WB infection affects oviposition and therefore potential population size of *M. quadrilineatus*, and numbers of infective individuals within populations. An additional way to explore

AY-WB affect on *M. quadrilineatus* population ecology, is to monitor nymph development on AY-WB infected plants versus uninfected plants. It is possible that AY-WB affects the development of nymphs. Faster or slower development may come at a cost to the insect and/or affect the dynamics of populations, or the numbers of infective individuals within a population. This would yield a clear picture of how AY-WB manipulates vector populations, and in turn increases its own dispersal.

AY-WB vector *M. quadrilineatus* belongs to the fascifrons or quadrilineatus-complex of sibling species (Kwon 1988; Hamilton 1983). It is plausible that these species diverged through speciation by separation in migratory routes, host selection and isolation. These sibling species are hard to distinguish and it is possible that vectors identified as *M. quadrilineatus*, may in fact be another species within the complex. *Macrostelus* leafhoppers like many other genera exhibit variation in morphology dependent on climate conditions, their fitness and season. The outbreak of AYP in watercress in Hawaii, initially assessed as vectored by *M. fascifrons*, then *M. quadrilineatus* and now thought to be *M. severini*, highlights the difficulty in identification of vectors in this complex. So far, *M. severini* and *M. fascifrons* are the only other known vectors of AYPs within the complex, however it is possible that others may vector phytoplasmas. To assess vector capacity of other sibling species within the complex may yield additional clues as to the divergence of these species. The vector efficiency of AY-WB to *Arabidopsis* could be compared between *M. severini*, *M. quadrilineatus* and *M. fascifrons*. This could yield clues as to differences between these three frequently confused leafhopper vectors and assess if AY-WB manipulations are effective in more than one leafhopper in the complex. AY-WB localization and titre at different stages of infection in *M. quadrilineatus* could be used as a benchmark to compare other sibling species within the quadrilineatus-complex. This would give valuable information on the phytoplasma incubation time in these different insects and any difficulties they encounter during their spread through

the insect. QRT-PCR assay of leafhopper defence genes, mediated by the Toll or innate mediated defence (Imd) pathways would be useful in comparison of defence response elicited by AY-WB infection within the leafhopper sibling species. This qRT-PCR approach could also be used in assessing *D. maidis* defence response to AY-WB infection.

AY-WB secreted protein SAP36 if found in high concentrations in *M. quadrilineatus* compared with *Arabidopsis* (MacLean *et al.* 2011). This suggests that SAP36 may play a role in AY-WB infection within the insect more than the plant. SAP36 protein could be purified and antibodies generated and used for localization of SAP36 within the *M. quadrilineatus*. This would yield clues as to the role of this protein within the insect. For example if it is located with phytoplasma within the gut epithelial cells or the salivary gland walls it may play a role in mediating cell responses to phytoplasma invasion. Or if found in the fat bodies, it may play a role in suppressing the defence response in haemolymph. Furthermore, if located in a particular area of the insect such as the salivary glands, these could be dissected out to generate a cDNA library, to be used in protein-protein interaction assays such as a yeast two-hybrid screen.

*D.5 Study of AY-WB impact on Arabidopsis and M. quadrilineatus, illuminates a potential web phytoplasma influenced insect-plant interactions.*

Use of the model *Arabidopsis* in this study is a powerful tool in fully understanding the complex of influences that phytoplasmas have on their host plants, insects and their interactions. AY-WB has an effect on its host plant *Arabidopsis* in terms of defence against herbivorous insects, and an effect on the fitness of its vector, in terms of fecundity. However, AY-WB influence over their hosts' fitness and defence is complicated by indirect effects, which affect populations of insect vectors, plants and the phytoplasma epidemiology.

This is a three-way interaction in which all parties have adopted strategies to survive and reproduce and disperse, which in turn, may by

perturbed by phytoplasma infection. In plants, *Arabidopsis* for instance, growth and development is influenced by phytoplasma infection, yellowing of young tissue, witches' broom, stunting, disturbed defences and so on, are all direct impacts on the plant, that have potential indirect effects on insect vector and phytoplasma ecology. Such direct and indirect effects cause a complex web of interactions that are dependent on state of the organisms involved, and have the potential to impact on the short and long term survival, reproduction and dispersal of all participants. The indirect effects of AY-WB disruption of plant development and defences are that *M. quadrilineatus* has higher fecundity on infected plants, and that AY-WB has an increased number of potential vectors in which to disperse to new plants. However, this is one thread in a web of effects.

AY-WB infection in *M. quadrilineatus* causes increased oviposition of the insect on healthy and infected *Arabidopsis*. This leads to a potentially larger population of vectors that will disperse phytoplasmas to new host plants, another thread of the web of effects.

The states of individual organisms within these interactions determine the complexity of the 'web of effects'.

There are 4 theoretical states of plant hosts to phytoplasmas defined as; immune, meaning the phytoplasma is unable to colonise the plant; resistant, indicating that the plant defends its self against widespread phytoplasma colonisation; susceptible, in which plants have a high titre of phytoplasmas and exhibit strong symptoms; and finally, tolerant, where the phytoplasma is present, but no symptoms of disease are visible in the plant (Purcell 1973). The same states could be defined in insect potential vectors to phytoplasmas; immune, the phytoplasma cannot complete its life cycle throughout the insect, so the insect is not a vector; resistant, the phytoplasma completes it's life cycle in the insect, but the insect vector defends itself to limit phytoplasma numbers; susceptible, phytoplasma completes its life cycle in the vector insect at a direct fitness cost to the insect; tolerant, the phytoplasma completes its life cycle in the vector at no direct cost to the insect.

Phytoplasmas themselves have virulence state, which (within interactions with their host plants and vectors) is a hypothetical trade off between virulence level and transmission. Virulence is the level of rapid reproduction and spread of phytoplasmas through the host, which can be costly and even pathogenic to host organisms. The trade-off hypothesis is the balance between rapidity of reproduction and spread throughout their hosts verses the risk of detriment and death of the host before the phytoplasmas can be transmitted to new hosts (Foissart *et al.* 2010, Messini *et al.* 2002, Marcone *et al.* 2010, Seemüller and Schneider, 2007).

The effects of all these states are complex but determine the nature of interactions between phytoplasmas, insect vectors and plant hosts. Thus they can give an indication of the future survival dispersal and success of phytoplasma species. A very simplified contingency table of these states is highlighted in here in table D.5.1. This figure shows the relationship between virulence of the phytoplasmas, the state of host insects and plants and how balance in virulence can be an evolutionary advantage.

		plant host			
		immune	resistant	susceptible	tolerant
insect vector	immune	no phytoplasma dispersal	no phytoplasma dispersal	no phytoplasma dispersal	no phytoplasma dispersal
	resistant	no phytoplasma dispersal	limited phytoplasma dispersal	limited phytoplasma dispersal	limited phytoplasma dispersal
	susceptible	no phytoplasma dispersal	limited phytoplasma dispersal	limited phytoplasma dispersal	phytoplasma dispersal
	tolerant	no phytoplasma dispersal	limited phytoplasma dispersal	limited phytoplasma dispersal	phytoplasma dispersal

		plant host			
		immune	resistant	susceptible	tolerant
insect vector	immune	no phytoplasma dispersal	no phytoplasma dispersal	no phytoplasma dispersal	no phytoplasma dispersal
	resistant	no phytoplasma dispersal	limited phytoplasma dispersal	phytoplasma dispersal	phytoplasma dispersal
	susceptible	no phytoplasma dispersal	limited phytoplasma dispersal	phytoplasma dispersal	phytoplasma dispersal
	tolerant	no phytoplasma dispersal	limited phytoplasma dispersal	phytoplasma dispersal	phytoplasma dispersal

**Table. D.5.1. Plant and insect host state can be influenced by phytoplasma virulence within hosts.** Insect and plant hosts can be defined as in 4 different states in response to phytoplasma infection; immune, so phytoplasmas cannot colonise; resistant, hosts mount defences to phytoplasma infection that impair phytoplasma spread; susceptible, hosts mount a defence or ineffective defence and the phytoplasma colonises; tolerant, hosts mount little or no defence to phytoplasma infection, and no

symptoms are apparent. The virulence of phytoplasmas plays an important role in the dispersal of phytoplasmas as it influences successful colonisation and spread of phytoplasmas within and between hosts that are resistant, susceptible and tolerant of phytoplasmas. Furthermore, phytoplasma virulence can influence the evolution of these states of their hosts over time if virulence is not balanced. Arrows highlight the direction of evolution between states of insect and plant hosts if phytoplasmas are avirulent or virulent in interactions with host insects and plants.

It is of note that if a tolerant insect or plant host has probably evolved to recognize the phytoplasma over time and adapted to not respond defensively. Moreover this recognition could evolve to immunity in future generations should the phytoplasma adopt greater virulence or avirulence (Purcell 1973). Therefore, phytoplasmas virulence versus transmission trade off is important in the evolution of phytoplasma interactions as well as the web of effects that result in the dispersal of phytoplasmas.

The impact of phytoplasma virulence level and insect and plant host states can be seen in many economically important phytoplasma interactions. For example, *Flavescence dorée* has a relatively high virulence on both its grapevine plant hosts and its vector *Scaphoideus titanus*, which affects longevity in both hosts (Bressan *et al.* 2005). I would hypothesize that the insect vector and the plant hosts are defending themselves against phytoplasma colonisation and therefore their states are either resistant or susceptible. These states and the virulence level of the phytoplasma not only impact directly on each party within an interaction, they have indirect effects that impact on the success of *Scaphoideus titanus* populations and grapevine productivity and the dispersal of the phytoplasma. The direct and indirect effects caused by interactions between phytoplasmas and their plant and insect hosts are further complicated by mixed infections. Mixed phytoplasma infections are found in many plant and insect hosts and their mixed levels of virulence have an impact on each other as well as their hosts. For example mixed strains of *C. Phytoplasma mali* or mixed strains of *C. P. prunorum* in fruit trees have various levels of

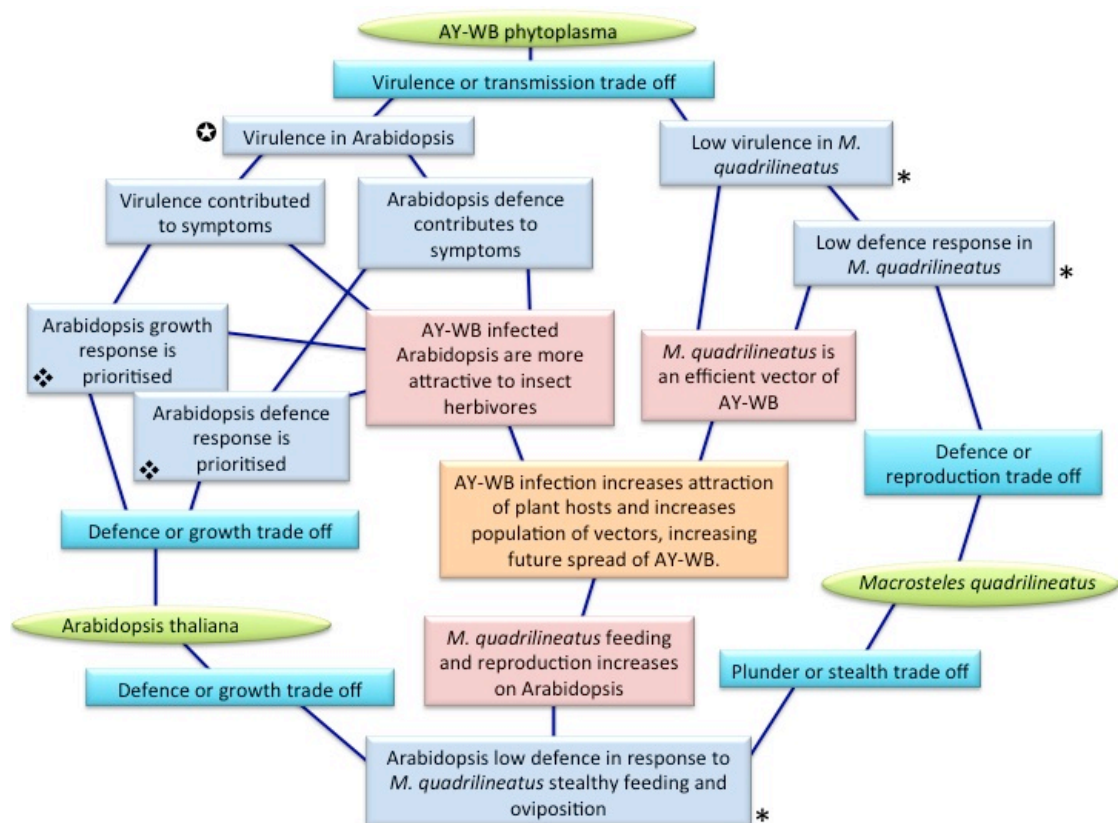
virulence. In addition the fruit tree hosts has different 'states' that determine defence response to each of these phytoplasma strains. If one strain is very virulent it affects the response of the plant to all strains. In addition, any high levels of virulence of strains in mixed infections may damage or kill the plant host earlier, thus narrowing the window of opportunity of transmission, thus the virulence or transmission trade off of phytoplasmas is further complicated (Seemüller and Schneider 2007, Marcone *et al.* 2010).

Use of a model system is key to unraveling the direct and indirect results of phytoplasma interactions with their host plants and insect vectors. In this study I used *Arabidopsis*, fully sequenced AY-WB phytoplasma and *M. quadrilineatus* to examine effects of interactions. The results here highlight that AY-WB phytoplasma alters *Arabidopsis* appearance and timing of senescence and renders the plants more attractive to *M. quadrilineatus*. In addition *Arabidopsis* defence response is compromised when infected with AY-WB. These alterations have indirect impacts on uninfected *M. quadrilineatus* fitness in terms of increased fecundity and survival. AY-WB infection within the vector *M. quadrilineatus* directly increases insect fecundity on uninfected *Arabidopsis*. From these data, it is sensible to hypothesize that *M. quadrilineatus* is tolerant of AY-WB infection and that *Arabidopsis* is either resistant or susceptible to AY-WB infections. Further investigation of the molecular defences in *Arabidopsis* will yield clues as to the defence response in the plant to AY-WB, thus identifying whether the insect is susceptible or resistant. AY-WB virulence in *Arabidopsis* may be higher than in *M. quadrilineatus* but it is unlikely to be significant, as plant longevity is not significantly affected.

The interactions between phytoplasmas and their hosts promote trade offs made by each partner in an interaction. For example the phytoplasma trade off is virulence or transmission (Froissart *et al.* 2010). Within the insect phytoplasmas cause a trade off between defence and reproduction, and within the plant a trade off between defence and growth (Purcell 1973, Nault 1990). It is likely that the

theoretical trade off between defence and reproduction in the *M. quadrilineatus* is settled on reproduction (Fig. D.5.1). The result of the trade off between defence and growth within *Arabidopsis* is not so clear as the symptoms of AY-WB infected plants indicate that either the virulence of AY-WB is sufficiently high to cause symptoms but the plant is putting energy into growth, or that the plant is defending itself and the defences are contributing to symptoms. Further study of *Arabidopsis* defence response to AY-WB will isolate results of these trade offs.

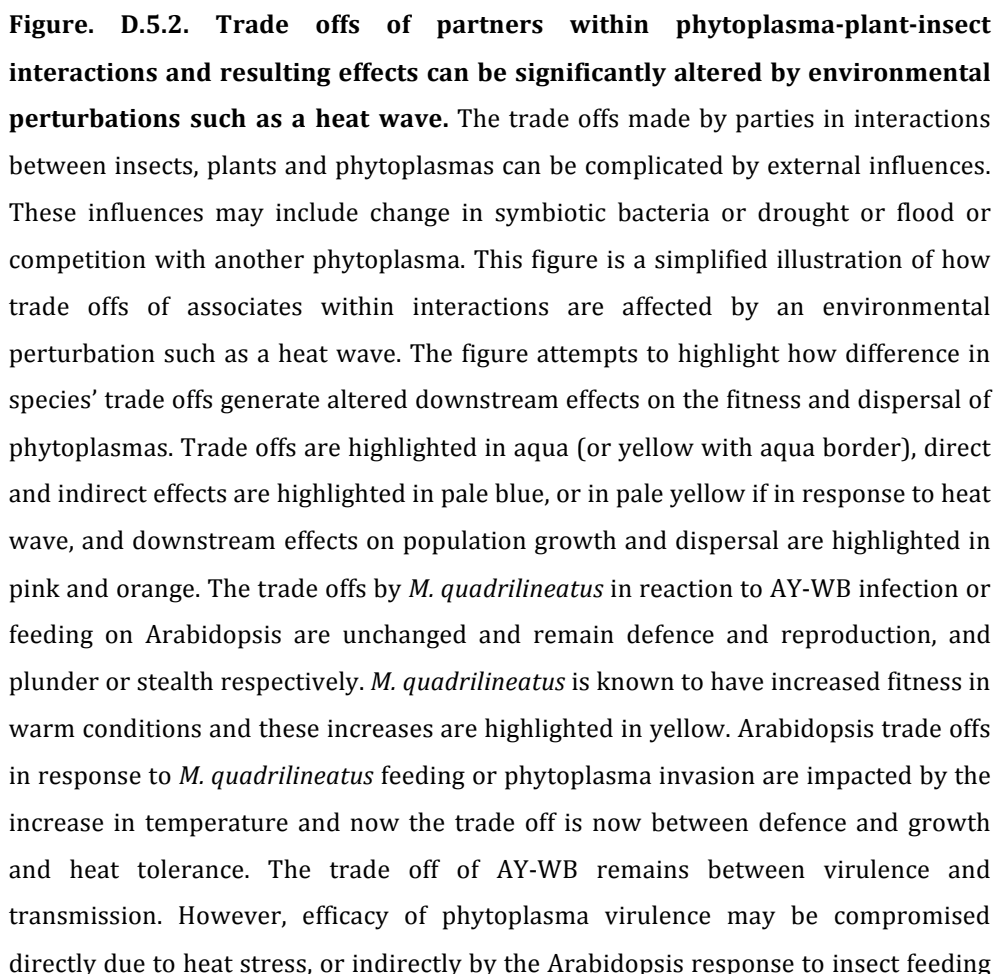
All results of these trade offs contribute to direct and indirect effects in a web of effects in phytoplasma-plant-insect interactions (Fig. D.5.1). these effects regulate The dissemination of phytoplasma in nature.



**Figure. D.5.1. Results of trade offs in partners within phytoplasma-plant-insect interactions contribute to the web of effects that determine phytoplasma dispersal.** The interactions between insects, plants and phytoplasmas prompt trade offs made by each associate in an interaction. This figure is a simplified illustration of how the trade offs of individual organisms within interactions, and their direct and indirect effects go on to affect population growth and dispersal of phytoplasmas.



Trade offs are highlighted in aqua, direct and indirect effects are highlighted in pale blue, and downstream effects on population growth and dispersal are highlighted in pink and orange. The trade off here by *M. quadrilineatus* is between defence and reproduction when challenged with AY-WB. When challenging Arabidopsis, *M. quadrilineatus* has another trade off between sequestration of nutrients from the plant and evasion of plant defence response (plunder or stealth). Arabidopsis responses to *M. quadrilineatus* feeding or phytoplasma invasion are governed by trade offs between defence and growth. Colonising phytoplasmas in either plant or insect hosts exhibit a trade off between virulence and transmission (the speed of reproduction and spread throughout the host versus the risk of the host dying before onward transmission is possible (due to severity of phytoplasma symptoms)). The result of the trade offs made by Arabidopsis and AY-WB in their interaction is not clear without further investigation. However it is clear that symptoms develop and that Arabidopsis don't have significantly reduced longevity after infection at the rosette stage (5 week old plants). This indicates that AY-WB in Arabidopsis is not excessively virulent and that Arabidopsis is either susceptible or resistant to AY-WB. Whilst symptoms are very clear, it is not yet known if Arabidopsis triggers a defence response to AY-WB infection, or whether any such defence response contributes to the phytoplasma symptoms in the plants❖. The virulence of AY-WB in Arabidopsis is not clear but it is not likely to be inordinate, as Arabidopsis don't show significant increase in mortality ⚡. AY-WB in *M. quadrilineatus* appears to have no negative effect on the insect. This indicates that AY-WB probably has low virulence in its' vector and that *M. quadrilineatus* mounts no significant defence response to infection\*. This would need to be further studied however to be confirmed. The over all long-term impacts on AY-WB phytoplasma fitness are; that populations grow and disperse by direct success in their hosts (Arabidopsis and vector *M. quadrilineatus*) and indirect generation of larger populations of vector leafhoppers.



plus heat stress plus AY-WB infection. Further investigations could yield clues as to the response that *Arabidopsis* makes in this situation and effect that has in phytoplasma virulence. Symptoms of AY-WB infected *Arabidopsis* are less severe than those seen on *Arabidopsis* with AY-WB and high temperatures (26°C). The overall impacts of heat wave occurrence on AY-WB phytoplasma fitness are; that population growth and dispersal in their hosts *Arabidopsis* and vector *M. quadrilineatus* is affected and that indirect generation of larger populations of vector leafhoppers may not occur.

The web on effects generated by this 3-way interaction results in the short-term effect of AY-WB infection is of benefit to the reproduction of *M. quadrilineatus* on *Arabidopsis*. This in the long term has a benefit to the more abundant next generation of leafhopper vectors. The effect of AY-WB infection on *Arabidopsis* is one of compromised defence to *M. quadrilineatus*, but this is likely to extend to other leafhopper herbivores, making the short-term a negative one. In addition, *Arabidopsis* appearance is altered which renders it either more apparent or more attractive to *M. quadrilineatus*. This may also translate to other leafhoppers and affect the numbers of herbivorous insects on *Arabidopsis*. If this is the case the negative short-term effect on *Arabidopsis* may be compounded.

In the long-term *Arabidopsis* future generations may adapt to be more resistant to AY-WB infection, leading to increased defence response in the plant and a knock on effect of virulence in the phytoplasma. It is probable that any negative effect may be confounded by environmental conditions such as increase in temperature or drought. *Arabidopsis* trade offs multiply under different conditions such as increased temperature. AY-WB-*Arabidopsis*-*M. quadrilineatus* interactions were studied under 22°C in 10 hour days or 26°C in 16 hour days, on AY-WB infected *Arabidopsis* in warmer conditions bolting and senescence occurred ca. 3-4 weeks earlier than in cooler conditions. This indicates that either the plant defences are overcome in warm conditions, or that AY-WB virulence is compromised in warmer conditions, or *Arabidopsis*

defends against one impact at a cost to the other, which causes premature aging and death of plant.

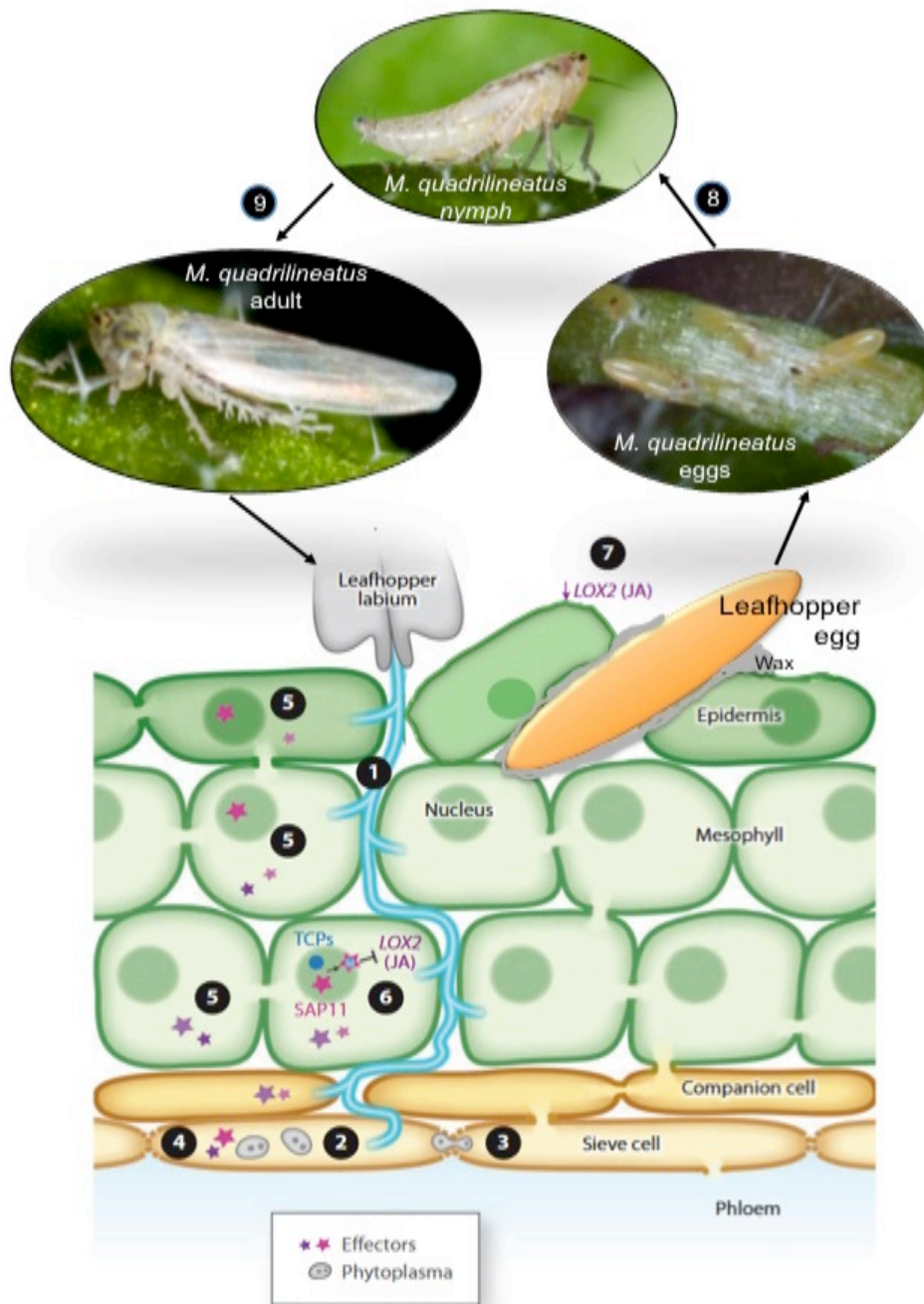
The long-term effects of AY-WB in cool conditions (22°C and 10 hour day), are marked by successful colonisation and reproduction in both hosts, together with indirect effects on insect vector population growth. Thus providing growing future populations of insect vectors within which to disperse.

*Arabidopsis*~AY-WB~*M. quadrilineatus* provides an invaluable model system with which to gain further understanding of how phytoplasma infection modulates hosts directly and indirectly and the web of effects on phytoplasma ecology. Use of this model also facilitates the study of phytoplasma epidemiology, effects of environmental perturbations and potential controls of phytoplasmas.

#### D.6 Summary

AY-WB infection in *Arabidopsis*, renders the plants more susceptible to *M. quadrilineatus* oviposition and *D. maidis* survival. Feeding of both leafhoppers is largely unaltered by AY-WB in the plant, suggesting that adult leafhoppers view the plants as favourable hosts. If a similar trend is noted on crops, this has far reaching consequences to the population numbers of vector leafhoppers, and non-host potential vectors, and the epidemiology of AY-WB. The secreted effector protein SAP11 is demonstrated to alter plant defence response to vectors by disrupting production of LOX2 and jasmonic acid. Thus, rendering the plant more susceptible to oviposition by vector *M. quadrilineatus* (Fig. D.4.1).

In addition, changes in plant morphology suggest that leafhoppers may be attracted to the light green lush new foliage of infected plants rather than healthy plants. Interestingly, it is not clear that AY-WB altered morphology attracts leafhoppers to feed and oviposit. If there is such an effect in crops it is unknown whether this is due to the allure of the plants or whether these plants have greater apparency that may lead host selection.



**Figure D.6.1. Model of phytoplasma use of secreted proteins in plants to alter phytoplasma dispersal by vectors.** This figure is adapted from Sugio *et al.* 2011b, original figure made by John Innes Centre, Norwich, UK. Photos taken by Andrew Davis in the insectary at the John Innes Centre, Norwich, UK. As the stylets move between plant cells to locate phloem (1), AY-WB are deposited with saliva into the phloem (2), AY-WB secrete effector protein SAP11 from the phloem (4), which targets plant cell nuclei (5) and destabilizes TCPs (6). Plant cells with reduced TCPs produce less LOX2 and synthesis of defence hormone jasmonic acid is reduced (6). This allows *M. quadrilineatus* to oviposit more eggs (7). Increased oviposition, increases the

number of nymphs produced on AY-WB infected plants (8), which in turn can vector AY-WB and become a larger number of vector adults in populations (9).

*M. quadrilineatus* is a leafhopper within an intriguing species complex, of which only 3 are known vectors of phytoplasmas. This begs the question; what are the underlying differences between the insects within this complex? The 3 known vectors may be more susceptible or tolerant to phytoplasma infection or they may be the only ones to have been challenged by phytoplasmas. Perhaps the others were resistant to virulent phytoplasmas and have evolved immunity in their history. Using the same model organisms with colonies of other *Macrostelus* species within this complex could yield valuable clues as to the evolution of AY-WB interaction with *M. quadrilineatus*.

This study is a solid start in understanding the phytoplasma manipulations of their host plants, which have direct and indirect effects on the success and dispersal of phytoplasmas. The model system of *Arabidopsis*~AY-WB~*M. quadrilineatus* provides a powerful tool in investigating the trade offs that each party in phytoplasma interactions make in their interactions. In addition, the short generation time of all three species allows for relatively rapid study of the direct and indirect effects of these trade offs and their knock on impact on phytoplasma ecology, and epidemiology.

This model system also provides a platform in which to examine genetics and proteomics of interactions together with the evolution of phytoplasma virulence and the coevolution of associations. Finally this model system provides a valuable interaction on which to study both environmental perturbations, with a view to examining the impact of climate change on phytoplasma epidemiology, and the potential phytoplasma control measures and products. This study invites further research into phytoplasma evolution, epidemiology and control utilising this precious model interaction system.



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### **Web references for figures**

R. Bourdeix<sup>1</sup>, K. Allou<sup>2</sup> and J. L. Konan Konan<sup>3</sup> Lethal Yellowing diseases of the coconut palm: an overview

[http://www.plantapalm.com/vpe/pestsndiseases/vpe\\_ly\\_coconut.htm](http://www.plantapalm.com/vpe/pestsndiseases/vpe_ly_coconut.htm)

(Figure. 1.)

<http://www.ars.usda.gov/pandp/people/people.htm?personid=1289>

Sourced 29/3/08. Web page of Dr Robert E. Davis. United States

Department of Agriculture, Molecular Plant Pathology. (Figure. 3.)